# Salt-inducible promoter derivable from a lactic acid bacterium, and its use in a lactic acid bacterium for production of a desired protein

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**(54) Title:** SALT-INDUCIBLE PROMOTER DERIVABLE FROM A LACTIC ACID BACTERIUM, AND ITS USE IN A LACTIC ACID BACTERIUM FOR PRODUCTION OF A DESIRED PROTEIN

**(57) Abstract**

The invention provides a salt-inducible promoter present in SEQ ID NO: 10 and derivable from a lactic acid bacterium in isolation from the coding sequence normally controlled by said promoter in a wild-type lactic acid bacterium, with modifications and important parts thereof. Also provided are a recombinant vector and a transformed lactic acid bacterium comprising such promoter, and the production of a desired protein by such transformed bacterium, whereby the gene encoding said desired protein or a precursor thereof is expressed under control of such promoter. The desired protein can be secreted by the bacterium due to the presence of a signal sequence. The action of the salt-inducible promoter is enhanced at a pH of about 4-4.5 and/or by the presence of glutamic acid. Such process can be used in a fermentation process, in which the desired protein is a lytic protein causing lysis of the cells and release of the cell content. Or the desired protein can be an enzyme involved in flavour formation, e.g. in producing dressings, spreads, sausages and sour dough, or a protein functioning in a cheese production process, such as chymosin or a precursor thereof, or an enzyme involved in cheese flavour formation.

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# SALT-INDUCIBLE PROMOTER DERIVABLE FROM A LACTIC ACID BACTERIUM, AND ITS USE IN A LACTIC ACID BACTERIUM FOR PRODUCTION OF A DESIRED PROTEIN

The invention relates to a salt-inducible promoter derivable from a lactic acid bacterium.

## Background of the invention and prior art

It is known that salt-inducible promoters exist in plants and cyanobacteria; the latter are rather specialised bacteria which can be used for nitrogen-fixation of soil as natural fertilisation and which taxonomically are quite separate from other bacteria.

Although inducible promoter systems are known in Gram-negative bacteria like *E. coli* and in the Gram-positive bacterium *Bacillus subtilis*, while recently in WO 95/31563 (Quest International B.V. (A. Nauta c.s.); see ref. 36) an inducible promoter system was described for lactic acid bacteria or their phages, no literature was found relating to salt-inducible promoters active in microorganisms apart from the above mentioned rather specialised cyanobacteria.

Although the expression "salt-initiated induction system" was present in said WO 95/31563 (Quest International B.V. (A. Nauta c.s.); ref. 36), no specific salt-initiated induction system was disclosed.

The present invention provides for the first time a salt-inducible promoter for lactic acid bacteria and its use in the production of polypeptides by lactic acid bacteria.

## Summary of the invention

The invention provides a salt-inducible promoter derivable from a lactic acid bacterium, in particular a salt-inducible promoter the nucleotide sequence of which is present in SEQ. ID. NO: 10 and in Figure 6. The advantage of a salt-inducible promoter active in lactic acid bacteria is first of all that salt is a natural food ingredient and therefore can be used as a food-grade inducer in food fermentation processes. For instance, during the salting stage of cheese curd various processes can be started when the higher salt concentration is used to trigger the formation of various proteins or peptides. Such processes include induced production and **secretion** of compounds which can contribute to the properties of the final cheese, e.g. enzymes and/or peptides that contribute to the formation of flavour compounds, and **lysis** of the lactic acid bacteria whereby peptidolytic enzymes are liberated by which the cheese ripening process is enhanced. Another process in which a salt-inducible promoter can be used advantageously is a process for the production of any protein or secondary metabolite by a food-grade microorganism, especially a lactic acid bacterium, whereby at the end of the culturing at a high cell density the microorganism is induced by a salt to produce the desired protein or secondary metabolite. As will be shown in the Examples, in this specification the term "salt" does only not mean common salt, i.e. sodium chloride, but also includes other halides like alkali metal, earth alkali metal and ammonium halides. In addition to the salt-induction by NaCl, KCl, $NH_4Cl$, $CaCl_2$, $MgCl_2$, NaI, and KI exemplified in Examples 1 and 2 (see also Table 3 below), it is envisaged that also other halides, i.e. bromides and perhaps fluorides, as well as halides with other cations such as substituted ammonium compounds, e.g. tetramethylammonium, or other metallic cations, e.g. $Al^{3+}$, will show the salt-inducing effect.

Still another process which can benefit from the presence of a salt-inducible promoter in a microorganism is the production of secondary metabolites, e.g. flavour or taste ingredients, *in situ* in a fermented product upon the addition of salt, examples of which include dressings and water-containing spreads, as well as sausages and sour dough.

The invention also provides a recombinant vector and a transformed lactic acid bacterium each comprising such a salt-inducible promoter.

## Brief description of the drawings

Figure 1.

Schematic representation of plasmid pORI13. ori$^+$, plus origin of pWV01; Em$^r$, erythromycin resistance gene; T, terminator; lacZ, promoterless *E. coli* ß-galactosidase gene fused to the ribosome binding site and translational start codon of the lactococcal ORF32 (see Example 1.1 below). Stop codons are indicated by asterisks.

Figure 2.

Scheme of the construction of plasmid pLS12 starting from plasmids pBSK+, pKL10, and pMG60 (see Example 1.1.4). The immediate result of the ligation of (1) the multiple cloning site of pBSK+ containing the Km$^r$ gene in the *Bam*HI site and (2) *Bam*HI-digested pLS11 is a precursor of pLS12 which is not depicted (see 1.1.4 below). After *Bam*HI-digestion of this precursor and self-ligation pLS12 is formed. This is indicated by the dashed arrows.

Figure 3.

Scheme of the construction of plasmid pLS13 starting from plasmids pORI28 and pLS12 (see Example 1.1.4).

Figure 4.

Scheme of the construction of plasmid pORI13 starting from plasmids pLS13, pMG60 and pORI28 (see Example 1.1.5).

Figure 5.

Deletion analysis NS3 (see Example 1.3). This shows the schematic drawings of the relevant parts of plasmids pNS3 (10 kb insert), pNS3d (2.4 kb insert), pNS3b (1.0 kb insert), pNS3e (0.5 kb insert), and pNS3f (0.4 kb insert).

Figure 6.

Nucleotide sequence of the NS3 locus and deduced amino acid sequences of the 3' end of *rnhB*, of *rggL*, and of the 5' end of *orfX* (see Example 1.5). Facing arrows, inverted repeats; rbs, ribosome binding site; -10 and -35 hexanucleotides are in boldface and underlined; vertical arrow, transcription start point. Stop codons are indicated with asterisks. A number of relevant restriction enzyme sites are shown.

Figure 7.
Alignment of the deduced translation products of *rggL* (see SEQ. ID. NO: 11) with *rgg* from *S. gordonii* (see ref. 27; M.C. Sulavik c.s.; 1992 and SEQ. ID. NO: 12) and part of ORF3, found downstream of the *L. lactis* C2 *pip* gene (see ref. 30; B.L. Geller c.s; 1993 and SEQ. ID. NO: 13). Asterisks indicate identical amino acids; conservative amino acids are indicated by dots (see Example 1.4).

Figure 8.
Alignment of the translation products of the 3' part of *rnhB* from *L. lactis* (see SEQ. ID. NO: 14) and *Vibrio cholerae* (U30472) (see SEQ. ID. NO: 15) and the complete *rnhB* genes from *E. coli* (see ref. 18; M. Itaya; 1990 and SEQ. ID. NO: 16) and *Haemophilus influenzae* (see ref. 33; R.D. Fleischmann c.s.; 1995 and SEQ. ID. NO: 17). The highest homology was found with *E. coli rnhB* (44% identical plus 13.9% similar amino acids). Asterisks, identical amino acids; periods, similar amino acids (see Example 1.4).

Figure 9.
Genomic organization of the NS3 locus. Prgg: *rggL* promoter, IR, inverted repeat, PNaCl: salt-inducible promoter (see Example 1.4).

Figure 10.
Sequence analysis NS3. The total *Pst*I-*Xba*I fragment given in Figure 6 is given here schematically with relevant restriction sites and the main features of the DNA. The indicated fragments were subcloned giving plasmids pNS3I - pNS3VI and pNS3i4 (see also Figure 27 and Example 1.4.1). Also primers used for sequencing are given.

Figure 11.
Autoradiogram of a northern blot of total RNA isolated from MG1363 or MGNS3i3 (*rggL*⁻) cells grown in the absence or presence of 0.5 M NaCl. The blot was hybridized with radioactively labeled *Xba*I-*Sau*3A fragment encoding the 5' end of *orfX*. The sizes of an RNA marker are given in the left margin (see Example 1.6).

Figure 12.
ß-galactosidase activity of NS3 as a function of the NaCl concentration in the culture medium. Samples were taken 7 hours after addition of NaCl to a 1:100 diluted culture (see Example 2).

Figure 13.
Nucleotide sequences of the fusion points of truncated *orfX* with either *lytPR* or *acmA* in pNS3PR **(Part A)** or pNS3AL3 **(Part B)**; see also SEQ. ID. NO: 18 and SEQ. ID. NO: 19, respectively and Examples 3 and 4. The translation of both genes is given below the DNA sequence. Translational stops are indicated with asterisk triplets. The transcription start site is shown by the vertical arrow, while the RBS's are in boldface. The *Xba*I site corresponds to position 1981 in Figure 4. The *Eco*RV and *Sca*I sites were used to make the fusions.

Figure 14.
Renaturing SDS-12.5% PAGE showing cell wall hydrolase activity in cell extracts prepared from samples taken 6 hours after addition of NaCl. Lanes: 1 and 2, LL108(pNS378); 3 and 4, LL108(pNS3PR); 5 and 6, LL108(pNS3AL3); 1, 3 and 5, no NaCl; 2, 4 and 6, plus NaCl. Molecular masses (in kilodaltons) of marker proteins are indicated on the left (see Examples 3 and 4).

Figure 15.
Optical density at 600 nm of LL108(pNS378)(squares) and LL108(pNS3PR)(triangles) grown in GM17. NaCl was added to

0.5 M end concentration to part of the culture at A600 = 0.5 (open symbols); see Examples 3 and 5.
N.B. In this specification the indication OD600 (optical density) is used instead of A600 (absorption).

Figure 16.
Scheme of the construction of plasmid pNS3PR starting from plasmid pIR1PR and a fragment containing the *rggL* gene and the salt-inducible promoter, which was amplified by PCR using primers NS3-7 and NS3-8 (see Table 2 below) and pNS3 as template (see Example 3.1).

Figure 17.
Optical density at 600 nm of LL108(pNS378)(squares) and LL108(pNS3AL3)(triangles) grown in GM17. NaCl was added to 0.5 M end concentration to part of the culture at A600 = 0.5 (open symbols); see Examples 4 and 5.

Figure 18.
Scheme of the construction of plasmid pNS3AL3 starting from plasmids pAL10, pORI28 and the fragment also described in Figure 16 (see Example 4.1).

Figure 19.
(A) SDS-12.5% PAA gel stained with Coomassie Briliant Blue of supernatant samples of *L. lactis* cultures taken 6 hours after addition of NaCl.
(B) Renaturing SDS-12.5% PAGE showing cell wall hydrolase activity. Lanes: 1 and 2, LL108(pNS378); 3 and 4, LL108(pNS3PR); 5 and 6, LL108(pNS3AL3); 1, 3 and 5, no NaCl added; 2, 4 and 6, plus NaCl. Molecular masses (in kilodaltons) of marker proteins are shown on the left. The arrow on the right indicates the position of pre-AcmA (see Example 5).

Figure 20.

PepXP activity in supernatants of cultures of MG1363*acmA*Δ1(pVE6007;pNS3PR), circles, MG1363*acmA*Δ1(pVE6007;pNS3AL3), triangles, and MG1363*acmA*Δ1(pVE6007;pNS378), squares. At t=0 (optical density at 600 nm=0.5) NaCl was added to a final concentration of 0.5 M (closed symbols) or 0.1 M (open symbols) to induce the expression of *lytPR*, *acmA*, or *lacZ*, respectively (see Example 5.1).

Figure 21.

PepXP activities in the supernatant fractions (black bars) and in the fractions obtained after hypo-osmotic wash of the cells (superimposed grey bars) of MG1363*acmA*Δ1(pVE6007) carrying pNS378, pNS3PR, pNS3AL3 or pGKAL1. Cells were induced with 0.025, 0.05, 0.1, 0.25, or 0.5 M of extra NaCl at an optical density of the cultures of 0.5 at 600 nm. As a negative control M17 containing 0.004 M NaCl was used. PepXP levels were determined two days after induction (see Example 5.1).

Figure 22.

Gene organization of the NS3 locus. Lollypop, inverted repeat; P, promoter. Relevant restriction enzyme sites are indicated (see Example 6). The black arrows indicate the positions of *lacZ* fusions in the chromosome of the indicated strains (*lacZ* not drawn to scale). The total sequences of the *gadR*, *gadC* and *gadB* genes are given in Figure 29 and SEQ. ID. NO: 21.

Figure 23.

Alignment of the deduced translation products of *gadC* from *L. lactis* and *gadC* from *S. flexneri* (Waterman and Small, 1996; ref. 47). Asterisks, identical amino acids; periods, similar amino acids. Putative membrane spanning domains are double underlined. Residues of a putative glutamate binding box are shown in a frame (see Example 6).

Figure 24.
Alignment of GadB from *L. lactis* MG1363, and glutamate decarboxylase from *Synechocystis* sp. strain PCC6803 (Kaneko *et al.*, 1996; ref. 44). Asterisks, identical amino acids; periods, similar amino acids (see Example 6).

Figure 25.
pH and glutamate-dependent expression of *gadC::lacZ*. Strain NS3 was grown in mM17 medium with (A) or without (B) 2% ß-glycerophosphate. ß-galactosidase activity was followed during growth (fat solid lines) either in the presence of 0.3 M NaCl (▲) or in the presence of 0.3 M NaCl plus 50 mM glutamic acid (■). Note that the scale of the left Y-axis in A and B is different. The optical density and pH of the cultures are indicated with dotted lines and thin lines, respectively (see Example 6).

Figure 26.
Schematic representation of the acid resistance mechanism in lactococci, involving the decarboxylation of glutamate to γ-aminobutyrate (see Example 6).

Figure 27.
Schematic representation of plasmids pNS3i4 (see Example 1.4.1), pNS3i5, pNS3i6 and pNS3i7 (for the latter three plasmids see Example 6).

Figure 28.
Schematic representation of plasmid pORI19S (see Example 1.6.1).

Figure 29.
Total nucleotide sequences of the *gadR*, *gadC* and *gadB* genes (see also SEQ. ID. NO: 21).

## Detailed description of the invention

The invention provides a salt-inducible promoter **derivable from a lactic acid bacterium**, in isolation from the coding

sequence which is normally controlled by said promoter in a wild-type lactic acid bacterium.
Such a salt-inducible promoter was found during the work resulting in the invention and described below in the Examples. This work yielded *inter alia* a DNA fragment originating from chromosomal DNA of *L. lactis* MG1363 in which a salt-inducible promoter appeared to be present.
The nucleotide sequence of this DNA fragment is given in Figure 6 and SEQ. ID. NO: 10.

Deletion analysis described in Example 1.3 - 1.3.1 revealed that salt-induction of the *lacZ* gene is still possible, when about 1 kb of genomic DNA isolated from *L. lactis* M~~1~~363 is present in front of the *lacZ* gene (which DNA is present in a *Hin*dIII-*Sau*3A fragment; see polynucleotide 1476-2426 in Figure 6). With shorter DNA fragments, i.e. containing only 540 bp (see the *Eco*RI-*Sau*3A fragment containing polynucleotide 1882-2422 in Figure 6) or only 440 bp (see the *Xba*I-*Sau*3A fragment containing polynucleotide 1982-2422 in Figure 6) upstream of the fusion point of the *lacZ* gene with the genomic DNA isolated from *L. lactis* M1363, salt-induction was only weak or not possible at all, respectively. Therefore, an essential part of this salt-inducible promoter seems to be the polynucleotide 1482-1925 of SEQ. ID. NO: 10, which is part of the *Hin*dIII fragment described in Example 1.3.1 below and comprises the 3' part of a gene indicated with *rggL* in Figure 6 up to and including the stop codon. It is believed that this part of the *rggL* gene can be combined with any promoter capable of driving expression of a structural gene in a lactic acid bacterium, but it is preferred that this part of the *rggL* gene is combined with the real promoter found in the genomic clone, which is the polynculeotide 1926-2000 including an inverted repeat (polynucleotide 1926-1967 of Figure 6) instead of a -35 region and including the -10 region (polynucleotide 1987-1992 of Figure 6), and with the further part of DNA upstream of the ORFX-encoding gene (see polynucleotide 2001-2068 of Figure 6).

Thus a preferred essential part comprises the polynucleotide 1482-2068 of SEQ. ID. NO: 10.
Since the best results were obtained with the about 2.4 kb *Pst*I-*Sau*3A DNA fragment described in Example 1.4, which contains the full *rggL* gene preceded by and under control of its own promoter, a salt-inducible promoter comprising the polynucleotide 1-2068 of SEQ. ID. NO: 10 is more preferred, and still more preferred is the use of the full 2.4 kb fragment, thus a salt-inducible promoter, which additionally comprises part of the ORF X gene together forming polynucleotide 1-2426 of SEQ. ID. NO: 10.
In addition to the various parts of the promoter just mentioned it is envisaged that also modifications thereof can be used according to the invention. Thus the invention also provides a modification of a salt-inducible promoter according to the invention or an essential part thereof, which comprises a DNA sequence essentially corresponding to a polynucleotide selected from the group consisting of

(a) polynucleotide 1482-1925 of SEQ. ID. NO: 10 followed by a promoter functional in a lactic acid bacterium,
(b) polynucleotide 1482-2068 of SEQ. ID. NO: 10,
(c) polynucleotide 1-2068 of SEQ. ID. NO: 10, and
(d) polynucleotide 1-2426 of SEQ. ID. NO: 10.

"Essentially corresponding to a polynucleotide" is understood as to include genetic variants, such as hybrid sequences containing a salt-inducible promoter or part thereof coupled to other homologous or heterologous DNA sequences including regulatory regions, and sequences containing modifications of the salt-inducible promoter or sequences having mutations, including mutations which still allow hybridization with the complementary strand of the salt-inducible promoter and genetic variants thereof, while still being capable of exerting the promoter function.

It is believed that the *rggL* gene, or in fact the rggL polypeptide, plays an important role in the effectiveness of the salt-inducible promoter present in the genome of *L. lactis* MG1363, isolated by the present inventors and used to transform other lactic acid bacteria in order to give them other desirable properties. But it is further believed, that not only the *rggL* gene indicated by polynucleotide 1095-1925 of SEQ. ID. NO: 10 will be functional in this respect, but also other DNA fragments encoding the same polypeptide, and even other DNA fragments encoding a modification of such rggL polypeptide still having the same or a similar functionality. Thus an aspect of the present invention is a DNA fragment capable of regulating a salt-inducible promoter active in a lactic acid bacterium, which comprises the polynucleotide 1095-1925 of SEQ. ID. NO: 10, or a modification thereof that (a) encodes the same polypeptide as said polynucleotide 1095-1925, or (b) encodes a modification of such polypeptide still having essentially the same regulating capacity.

A further aspect of the invention is a recombinant vector comprising a salt-inducible promoter or an essential part thereof as described above, or a DNA fragment capable of regulating a salt-inducible promoter active in a lactic acid bacterium as described above in combination with a DNA fragment selected from the group consisting of a DNA fragment containing the polynucleotide 1926-2000 of SEQ. ID. NO: 10 and modifications thereof still having essentially the same promoting capacity.

The invention also provides a transformed lactic acid bacterium comprising a salt-inducible promoter or an essential part thereof as described above, or a DNA fragment capable of regulating a salt-inducible promoter active in a lactic acid bacterium as described above in combination with a DNA fragment selected from the group consisting of a DNA fragment containing the polynucleotide 1926-2000 of SEQ. ID. NO: 10 and modifications thereof still having essentially the same promoting capacity. The promoter, essential parts thereof and other DNA fragments as described above are preferably present in the chromosome of the lactic acid bacterium, but they can also be present as part of a plasmid that can be maintained during growth of the lactic bacterium.

The lactic acid bacterium containing a salt-inducible promoter according to the invention either can be the natural host from which the salt-inducible promoter is derivable, or it can be a different lactic acid bacterium. If both the lactic acid bacterium and the salt-inducible promoter applied according to the invention are the same as in the natural situation, the lactic acid bacterium is transformed by incorporating one or more DNA fragments, or the salt-inducible promoter, originating from a lactic acid bacterium, is used in isolation from the coding sequence which is normally controlled by said promoter in a wild-type lactic acid bacterium.

Further the invention provides a process for the production of a desired protein by a transformed lactic acid bacterium, whereby the gene encoding said desired protein or a precursor thereof is expressed under control of an inducible promoter, characterised in that the promoter is a salt-inducible promoter or an essential part thereof according to the invention or a DNA fragment capable of regulating a salt-inducible promoter active in a lactic acid bacterium as described above in combination with a DNA fragment selected from the group consisting of a DNA fragment containing the polynucleotide 1926-2000 of SEQ. ID. NO: 10 and modifications thereof still having essentially the same promoting capacity. Preferably the transformed lactic acid bacterium is food-grade due to the use of food-grade DNA sequences and/or removal of non-food-grade DNA sequences.
For some embodiments it is desirable that the desired protein is secreted by the lactic acid bacterium due to the presence of a DNA fragment fused to the gene encoding the desired protein and effecting secretion of the desired protein or a precursor thereof.
A process according to the invention using a salt-inducible promoter for the expression of a desired gene can be used in a fermentation process, in which the desired protein is a lytic protein causing lysis of the bacterial cells so that the contents of the cells can be released, or in a

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or source |
| --- | --- | --- |
| **Strains** | | |
| *L. lactis* | | |
| MG1363 | Plasmid-free derivative of NCDO712 | ref. 5 |
| MG1363*acmA* $\Delta$1 | AcmA$^-$ derivative of MG1363 | ref. 31 |
| LL108 | *repA*$^{\pm}$ derivative of MG1363, $Cm^r$ | This work and ref. 40 |
| LL302 | *repA*$^{\pm}$ derivative of MG1363 | This work and ref. 40 |
| NS3 | NS3::*lacZ*, $Em^r$ | This work |
| MGNS3i3 | $\Delta$*rggL* derivative of MG1363, $Sp^r$ | This work |
| *E. coli* | | |
| MC1000 | $F^-$, *ara* D139 (*ara ABC-leu*)7679, *galU, galK, LacX74, rspL, thi* | ref. 7 |
| EC1000 | *repA*$^{\pm}$ derivative of MC1000, $Km^r$, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene | This work and ref. 39 |
| EC101 | *repA*$^{\pm}$ derivative of JM101, $Km^r$ | ref. 37 |
| NM522 | supE, thi, $\Delta$(lac-proAB), *$\Delta$hsd5* ($r^-$,$m^-$), [F',*proAB, lacI*$^q$*Z $\Delta$M15*] | ref. 6 |
| **Plasmids** | | |
| pMG60 | $Em^r$, fusion of promoter 32 with *lacZ* | ref. 24 |
| pORI28 | $Em^r$, $Ori^+$ of pWV01, $Rep^-$ | This work and ref. 40 |
| pLS28 | $Em^r$, '*lacZ* of pMG60 in pORI28 | This work |
| pKL10 | $Em^r$, carrying the pBR322 ori | ref. 17 |
| pLS10 | pKL10, carrying the 5'-end of *lacZ* from pMG60 | This work |
| pLS11 | pKL10, carrying the complete *E. coli lacZ* gene | This work |
| pUC7K | $Ap^r$, $Km^r$ | ref. 11 |
| pBSK+ | $Amp^r$, $\alpha$-lacZ | ex Stratagene |
| pLS12Km | $Em^r$, $Km^r$, pLS11 carrying the multiple cloning site of pBSK+ | This work |
| pLS12 | $Em^r$, derivative of pLS12Km | This work |
| pLS13 | $Em^r$, promoterless *lacZ* | This work |
| pORI13 | Promoterless *lacZ*, $Em^r$, $Ori^+$ of pWV01, $Rep^-$ | This work |
| pVE6007 | $Cm^r$, pWV01 derivative encoding a temperature sensitive Rep protein | ref. 28 |

TABLE 1 (continued) Bacterial strains and plasmids

| Plasmids (continued) | Relevant characteristics | Reference or source |
|---|---|---|
| pNS3 | Em$^r$, NS3::*lacZ*, carries a 10 kb *Sau*3A chromosomal DNA fragment | This work |
| pNS3b | Em$^r$, NS3::*lacZ*, carries a 1.0 kb *Hin*dIII-*Sau*3A chromosomal DNA fragment | This work |
| pNS3d | Em$^r$, NS3::*lacZ*, carries a 2.5 kb *Pst*I-*Sau*3A chromosomal DNA fragment | This work |
| pNS3e | Em$^r$, NS3::*lacZ*, carries a 540 bp *Eco*RI-*Sau*3A chromosomal DNA fragment | This work |
| pNS3f | Em$^r$, NS3::*lacZ*, carries a 440 bp *Xba*I-*Sau*3A chromosomal DNA fragment | This work |
| pNS378 | Em$^r$, NS3::*lacZ*, carries a 1280 bp PCR fragment amplified with NS3-7 and NS3-8 | This work |
| pUC19 | Ap$^r$, *lacZ'* | ref. 8 |
| pNS3I | Ap$^r$, pUC19 with a 401 bp *Eco*RI-*Hin*dIII fragment of pNS3b | This work |
| pNS3II | Ap$^r$, pUC18 with a 784 bp *Xba*I fragment of pNS3 | This work |
| pNS3III | Ap$^r$, pUC18 with a 470 bp *Xba*I fragment of pNS3 | This work |
| pNS3IV | Ap$^r$, pUC18 with a 604 bp *Hin*dIII fragment of pNS3 | This work |
| pNS3V | Ap$^r$, pUC18 with a 743 bp *Eco*RI-*Xba*I fragment of pNS3 | This work |
| pNS3VI | Ap$^r$, pUC19 with a 833 bp *Hin*dIII-*Pst*I fragment of pNS3 | This work |
| pORI19 | Em$^r$, $\alpha$-*lacZ*, Ori$^+$ of pWV01, Rep$^-$ | ref. 37 |
| pNS3i4 | Em$^r$, pORI19 with a 561 bp *Sau3A*-*XbaI* chromosomal DNA fragment | This work |
| pORI19S | Sp$^r$, $\alpha$-*lacZ*, derivative of pORI19 in which the *Stu*I-*Xmn*I fragment was replaced by an *Eco*RV-*Sma*I fragment carrying the Spectinomycin resistance gene | Lab. collection |
| pNS3i3 | Sp$^r$, internal *Xba*I-*Hin*dIII fragment of *rggL* in pORI19S | This work |
| pIR1PR | Em$^r$, phage r1-t *lytPR* fused to r1-t regulatory cassette | ref. 35 |
| pNS3PR | Em$^r$, NS3::*lytPR* | This work |
| pAL10 | Ap$^r$, *acmA* $\Delta$*Sac*I | ref. 34 |
| pAL101 | Ap$^r$, derivative of pAL10, *Bgl*II sites deleted | This work |

TABLE 1 (continued) Bacterial strains and plasmids

| Plasmids (continued) | Relevant characteristics | Reference or source |
|---|---|---|
| pAL102 | $Ap^r$, $Em^r$, $Ori^+$, derivative of pAL101 | This work |
| pNS3AL3S | $Em^r$, NS3::*acmA* Δ*Sac*I, derivative of pAL102 | This work |
| pNS3AL3 | $Em^r$, NS3::*acmA* | This work |

fermentation process, in which the desired protein is an enzyme involved in the *in situ* production of secondary metabolites as flavour or taste ingredients. Examples of end products include dressings and water-containing spreads, as well as sausages and sour dough.

Still another process which can benefit from the presence of a salt-inducible promoter in a microorganism is a fermentation process, in which the desired protein is a protein having a function in a cheese production process, such as chymosin or a precursor thereof, or an enzyme involved in cheese flavour formation.

The invention is exemplified by the following Examples 1-6. preceded by a description of the materials and methods that were used.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions**

The bacterial strains and plasmids used in the work described are listed in Table 1 above.

*L. lactis* was grown at 30°C in M17 medium, with 0.5% glucose; solidified M17 medium contained 1.5% agar. Erythromycin (Em) and chloramphenicol (Cm) were used at final concentrations of 5 µg/ml, spectinomycin (Sp) was used at 100 µg/ml. 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) was used at a final concentration of 0.008%.

*E. coli* was grown in TY broth at 37°C with vigorous agitation or on TY medium supplemented with 1.5% agar. Ampicillin (Ap) and Em were used at 100 μg/ml, Sp at 50 μg/ml.

**Molecular cloning techniques**

DNA and RNA techniques were performed essentially as described by Sambrook c.s. (see ref. 16). DNA was introduced by electrotransformation in *E. coli* (see ref. 19; E.R. Zabarovsky & G. Winberg; 1990) and in *L. lactis* (see ref. 15; H. Holo & I.F. Nes; 1989). DNA sequencing was done on double-stranded plasmid DNA by the dideoxy chain-termination method (see ref. 4; F. Sanger c.s.; 1977) and the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the manufacturer's instructions.

Several oligonucleotides prepared and used in the work described are listed in Table 2.

TABLE 2. Oligonucleotides used in this work

| Name | Nucleotide sequence (5' to 3') | |
|---|---|---|
| NS3-1 | CATTGAGATA ATCAGATAC (19) | SEQ. ID. NO: 1 |
| NS3-2 | GCAGAGATTG GGGAAG (16) | SEQ. ID. NO: 2 |
| NS3-5 | GGGGCCCTCT CTTATGTGTT AAATTTTCAG GCGC (34) | SEQ. ID. NO: 3 |
| NS3-6 | ATATCGTTCA CGTTTTCC (18) | SEQ. ID. NO: 4 |
| NS3-7 | GCGATATCCA GTACTTCATC ATACCTCCTT ATATTTATGA TTG (43) | SEQ. ID. NO: 5 |
| NS3-8 | GCGAGCTCAG ATCTGAGCGT TGTATAAGCT TTTATGTCTT TC (42) | SEQ. ID. NO: 6 |
| NS3-9 | GTTTGACTGA CCCAAC (16) | SEQ. ID. NO: 7 |
| NS3-10 | CCGCTTCAAT GGTTTTG (17) | SEQ. ID. NO: 8 |
| NS3-11 | CAGTCAAAAC CATTGAAGCG GTTAATGCGA AAAAACCG (38) | SEQ. ID. NO: 9 |

The oligonucleotides were synthesized with an Applied Biosystems 392A DNA synthesizer (Applied Biosystems Inc. Foster City, CA). DNA sequences were analyzed with the PC/Gene sequence analysis program (IntelliGenetics Inc., Geneva, Switzerland). Protein homology searches against the Genbank were carried out using the FASTA program (see ref. 12; W.R. Pearson & D.J. Lipman; 1988). Protein sequence alignments were carried out with the PALIGN program of PC/Gene using the structure genetic matrix or with the CLUSTAL program, both with standard settings.

In this specification the following endonuclease restriction sites are used:

| giving staggered ends | | giving blunt ends | |
|---|---|---|---|
| *Apa*I | GGGCC↓C | *Eco*RV | GAT↓ACT |
| *Bam*HI | G↓GATCC | *Pvu*II | CAG↓CTG |
| *Bgl*II | A↓GATCT | *Sca*I | AGT↓ACT |
| *Bss*HII | G↓CGCGC | *Sma*I | CCC↓GGG |
| *Cla*I | AT↓CGAT | *Ssp*I | AAT↓ATT |
| *Eco*RI | G↓AATTC | *Stu*I | AGG↓CCT |
| *Hind*III | A↓AGCTT | *Xmn*I | GAANN↓NNTTC |
| *Mlu*I | A↓CGCGT | | |
| *Not*I | GC↓GGCCGC | | |
| *Pst*I | CTGCA↓G | | |
| *Sac*I | GAGCT↓C | | |
| *Sal*I | G↓TCGAC | | |
| *Sau*3A | ↓GATC | | |
| *Spe*I | A↓CTAGT | | |
| *Sph*I | GCATG↓C | | |
| *Taq*I | T↓CGA | | |
| *Xba*I | T↓CTAGA | | |
| *Xho*I | C↓TCGAG | | |

**ß-Galactosidase assays**

Cell extracts were prepared from exponentially growing cultures. ß-Galactosidase activity was determined as described by Miller (see ref. 2; J.H. Miller; 1972). Protein concentrations in the cell extracts were determined by the method of Bradford (see ref. 3; M.M. Bradford; 1976) with bovine serum albumin as a standard.

**Example 1**

**Isolation of a salt-inducible promoter**

Several plasmids and transformed strains had to be prepared before a salt-inducible promoter could be isolated. Thus first some helper strains were constructed containing the *repA* gene of pWV01, i.e. *L. lactis* LL108 and *L. lactis* LL302 (see 1.1.2 below) and *E. coli* EC1000 (see 1.1.1 below). This work is based on the technology described in EP-A1-0 487 159 (see ref. 26; Unilever N.V./PLC (C.J. Leenhouts c.s.); 1992). Subsequently several plasmids were constructed:

- plasmid pORI28 with plasmids pTC2, pUK24 and pORI24 as intermediates (see 1.1.3 below),
- plasmid pLS13 with plasmids pLS10, pLS11, pLS12 and pORI28 as intermediates (see 1.1.4 below),
- the promoter screening vector plasmid pORI13 with plasmids pORI28, pLS28 and pLS13 as intermediates using lactococcal helper strains LL108 and LL302 (see 1.1.5 below).

These plasmids carry the ORI$^+$ of pWV01 but lack the *repA* gene for the replication initiation protein. However, they can be replicated in the helper strains containing the *repA* gene of pWV01.

**1.1 A lactococcal genome bank in pORI13**

To be able to assess expression of chromosomal genes, the promoter screening vector **pORI13 was constructed** (Figure 1). To allow only transcriptional fusions one stop codon in each reading frame was present immediately upstream of the *E. coli lacZ* gene. The *lacZ* gene is preceded by lactococcal translation signals derived from ORF32 (see ref. 24; M. van de Guchte c.s.; 1991 and ref. 10; J.M.B.M. van der Vossen c.s.; 1987). Since plasmid pORI13 carries the ORI$^+$ of pWV01 but lacks the *repA* gene for the replication initiation protein, it can be used for Campbell-type integrations. Random *Sau*3A fragments obtained from total chromosomal DNA of *L. lactis* MG1363, ranging in size from 1 to 10 kb, were cloned in the *Bam*HI site of pORI13. With the ligation mixture 48 EC1000 transformants were obtained and checked for their plasmid content. About 73% of these contained pORI13 with a chromosomal insert while the remainder contained pORI13. The average insert size was 3.3 kb. The restriction enzyme digestion pattern was different for all 48 clones. Plasmid DNA was isolated from 2,460,000 pooled EC1000 transformants (see 1.1.6 below) and used to transform *L. lactis* MG1363(pVE6007). After the temperature-up shift to force integration of the pORI13 derivatives, 9000 Em$^r$ clones were obtained by plating on selective sucrose GM17 plates containing X-gal and 0.3 M NaCl at 37°C for 16 hours resulting in 195 colonies that stained blue after prolonged incubation at 30°C (see 1.1.7 below).

**1.1.1 Construction of *E. coli* EC1000, being a *repA*$^+$ derivative of MC1000, Km$^r$, and carrying a single copy of the pWV01 *repA* gene in the *glgB* gene**

The *repA* gene from pWV01 was introduced onto the chromosome of *E. coli* MC1000 as described for JM101 by Law c.s. (see ref. 37; J. Law c.s.; 1995). pKVB2 (see ref. 37 and ref. 11; J.A.K.W. Kiel c.s.; 1987) is a Tc$^r$ Km$^r$ plasmid of 11.7 kb containing the origin of replication of pBR322. It carries the *E. coli* chromosomal *glgB* gene in which an internal 1.2 kb *Bam*HI fragment was replaced by the Km$^r$ gene from the *Streptococcus faecalis* plasmid pJH1. The *repA* gene from pWV01, driven by the lactococcal consensus promoter P23, was taken as a *Hin*dIII-*Pvu*II fragment from pUC23rep3 (see ref. 37 and ref. 22; K.J. Leenhouts c.s.; Sept. 1991) and used to replace a 700-bp *Sma*I fragment within *glgB* without interrupting the Km$^r$ gene. The resultant plasmids pEC1 and pEC2 differ solely in the orientation of *repA* (see ref. 37; J. Law c.s.; 1995).

Plasmids pEC1 and pEC2 were used to transform *E. coli* JM101. Before plating on selective media the transformation mixtures were transferred for 30 generations in the absence of antibiotic and then plated on Km-containing plates. Colonies were tested for glycogen production. Non-glycogen producing colonies were transferred onto plates containing Km and Tc and onto plates containing Km alone. Km$^r$ Tc$^s$ colonies were found and were plasmid-free and contained *repA* integrated at the specific site on the chromosome. Confirmation of the RepA$^+$ nature of one of the strains (*E. coli* EC1000) was obtained by the successful transformation of this strain with an ORI$^+$ RepA$^-$ plasmid. This work will also be described in a publication by Leenhouts c.s. accepted by Mol. Gen. Genet. for publication in 1996 (see ref. 39; K. Leenhouts c.s.; 1996).

**1.1.2 Construction of *RepA$^+$* *L. lactis* strains LL108 and LL302**

Two integration plasmids carrying pWV01 *repA*, pKL15A and pUK30, were constructed.
Plasmid pKL15A is a derivative of the pBR322-based Campbell-type integration plasmid pHV60 (see ref. 14; K.J. Leenhouts; 1989) in which the *repA* gene from plasmid pUC23rep3 (see ref. 22; K.J. Leenhouts; Sept. 1991) was inserted. Integration of pKL15A into the chromosome of *L. lactis* MG1363 by selection for chloramphenicol resistance **resulted in *L. lactis* strain LL108** carrying approximately 15 tandem copies of the integration plasmid, as has been described earlier for similar plasmids (see ref. 14; K.J. Leenhouts; 1989).
The replacement-type integration vector pUK30 was obtained by cloning the *repA* fragment of pUC23rep3 in the multiple cloning site of pUK29. The latter plasmid is a derivative of pUK21 (see ref. 23; J. Vieira & J. Messing; 1991) and carries the Em resistance gene of pUC19E (see ref. 17; K.J. Leenhouts; 1990) in the *Xho*I site, the 3'-end of the *L. lactis pepXP* gene (see ref. 20; B. Mayo c.s.; 1991) as a 1.5 kb *Xba*I fragment in the *Xba*I site, and the 5'-end of *pepXP* as a 1.5 kb *Spe*I-*Mlu*I fragment in the *Bgl*II and *Sph*I sites.
Plasmid pUK30 was used in a two-step gene-replacement strategy (see ref. 21; K.J. Leenhouts c.s.; Aug. 1991) for transforming *L. lactis* MG1363 **yielding *L. lactis* strain LL302**. This strain contained one copy of pWV01 *repA* inserted in the *pepXP* gene. Both strain LL302 and strain LL108 allow the replication of pWV01-based vectors which lack *repA*. This work will also be described in a publication by Leenhouts c.s. submitted for publication in 1996 (see ref. 40).

**1.1.3 Construction of pORI28**

The $Tc^r$ of pLS1 (see ref. 9; S.A. Lacks; 1986) was introduced in the *Sma*I site of pMTL25 (see ref. 13; S.P. Chambers c.s.; 1988) **to produce pTC2**. This Tc resistance gene was isolated from plasmid pTC2 as a 1.6 kb *Bam*HI fragment and plasmid pUK21 (see above) was digested with *Xho*I. Prior to ligation of the two fragments, blunt ends were generated by Klenow enzyme treatment. The ligation **resulted in pUK24**, in which the $Tc^r$ gene is flanked by two *Xho*I restriction sites. The 1.7 kb *Spe*I fragment of pUK24 carrying the $Tc^r$ gene was treated with Klenow enzyme to create blunt ends. A 601 bp *Taq*I fragment of pWV01 which carries the plus origin of replication ($Ori^+$) but lacks the gene encoding the replication initiation protein (*repA*), was also treated with Klenow enzyme and both fragments were ligated, **resulting in pORI24**. The *Xho*I fragment of pORI24 carrying the $Tc^r$ gene was replaced by the $Em^r$ gene from plasmid pUC19E, located on a 1 kb *Sal*I fragment, which **resulted in plasmid pORI28**. This work will also be described in a publication by Leenhouts c.s. accepted by Mol. Gen. Genet. for publication in 1996 (see ref. 39; K. Leenhouts c.s.; 1996).

**1.1.4 Construction of pLS13**

The *lacZ* gene of pMG60 (see ref. 24; M. van de Guchte c.s.; 1991) was cloned in pKL10 (see ref. 17; K.J. Leenhouts; 1990) in two steps. In the first step the 1250 bp *Ssp*I fragment ex pMG60, containing the 5'-end of *lacZ* gene and the lactococcal RBS of ORF32, was ligated with the Klenow treated *Xba*I site of pKL10. **The resulting construct, pLS10**, was restricted with *Hin*dIII, the *Hin*dIII sticky ends were made blunt and then the fragment was treated with *Cla*I. Subsequently, the resulting fragment was ligated with a *Cla*I-*Xmn*I fragment of pMG60 containing the additional part of the *lacZ* gene, including a transcriptional terminator, **resulting in pLS11.**

In the next step the multiple cloning site (mcs) of pBSK+ had to be inserted upstream of the RBS preceding the *lacZ* gene. To be able to select the mcs of pBSK+ it was marked by cloning a *Bam*HI fragment from pUC7K, coding for a $Km^r$ gene,

into the *Bam*HI site of the mcs. Subsequently the mcs was excised from this plasmid using *NotI* and *XhoI*. The sticky ends were made blunt-ended and the fragment was ligated into the *Bam*HI site (also made blunt-ended) of pLS11 in front of the *lacZ* gene. Finally the $Km^r$ gene was deleted using *Bam*HI followed by self ligation. **This resulted in the integration expression vector pLS12** (see Figure 2).
The erythromycin resistance gene of pLS12 was replaced by that from pORI28, including a multiple cloning site by isolating a *Stu*I-*Xba*I fragment from pORI28. This fragment was ligated into pLS12 digested with *Eco*RI, made blunt-ended with Klenow enzyme and subsequently digested with *Xba*I. The **resulting construct was designated pLS13** (see Figure 3).

**1.1.5 Construction of pORI13**

For the construction of a promoter screening vector, the *E. coli lacZ* gene fused to lactococcal translation signals, as present on pMG60 (see ref. 24; M. van de Guchte c.s.; 1991), was used. The *lacZ* gene was placed in the integration vector pORI28 in two steps (see ref. 40; K.J. Leenhouts; submitted for publication 1996).
In the first step, a 2.5 kb *Cla*I-*Xmn*I fragment of pMG60 was ligated in *Xba*I-*Cla*I linearized pORI28 of which the XbaI site was made blunt using Klenow. The ligation mixture was digested with *Eco*RI to prevent replication of pMG60 and used to transform the $RepA^+$ lactococcal helper strain LL108. **The resulting construct, pLS28,** was cut with *Bgl*II and *Bss*HII. The 5' end of the *lacZ* gene was liberated from pLS13 using the same restriction endonucleases. This 1.6 kb fragment was ligated to pLS28 and the ligation mixture was used to transform the $RepA^+$ *L. lactis* helper strain LL302. **The resulting plasmid was designated pORI13** (see Figure 4).

**1.1.6 Construction of a genome bank in pORI13**

Total chromosomal DNA of *L. lactis* MG1363 was partially digested with *Sau*3A to obtain fragments ranging in size from 1 to 10 kb which were ligated to *Bam*HI and alkaline phosphatase treated pORI13. The chromosomal fragments were ligated in the linearized pORI13. This ligation mixture was used to transform the $RepA^+$ *E. coli* helper strain EC1000 (see 1.1.1 above). Transformants were collected from agar plates by pouring 2 ml of TY broth on each plate, and their plasmid DNA was isolated.

**1.1.7 Integration of the bank in the *L. lactis* genome**
The plasmid mixture obtained in the previous step was used to transform *L. lactis* MG1363(pVE6007). After electroporation, cells were suspended in recovery medium of 30°C (see ref. 15; H. Holo & I.F. Nes; 1989) . After 1.5 hour 5 μg/ml erythromycin was added and incubation at 30°C was prolonged for 0.5 hour. Cells were shifted to 37 °C for 2 hours and then plated on sucrose (0.5 M) GM17 agar containing X-gal, erythromycin and 0.3 M NaCl. Incubation was at 37°C for 24 hours and subsequently at 30°C. The recovery of pORI13 derivatives from the chromosome of selected integrants was done as described by Law c.s. (see ref. 37; J. Law c.s.; 1995).

**1.2 Identification of a clone expressing *lacZ* in a salt-dependent way**
Colonies expressing ß-galactosidase in the presence of NaCl were transferred to GM17 agar plates with or without 0.5 M NaCl. Of the 195 selected blue colonies (see 1.1 above) 80 were white on the NaCl-free plates, indicating the absence of ß-galactosidase expression. The intensity of the blue colour in the presence of NaCl was similar for all 80 clones. The integrated pORI13 derivatives in 5 of these clones, called NS1-NS5, were rescued and appeared to be identical at the restriction enzyme level. All five plasmids expressed the salt-dependent phenotype. One of these, pNS3 isolated from clone NS3, was selected for further characterization.

**1.3 Deletion analysis of the genomic region of NS3**
Restriction enzyme analysis of pNS3 revealed that about 10 kb of chromosomal DNA had been cloned. A number of restriction enzyme sites was used to map the salt-dependent promoter. A

*Pst*I deletion (pNS3d) showed that NaCl-dependent *lacZ* expression is linked to a 2.4 kb fragment directly upstream of the *Sau*3a site at the original fusion point (see Figure 5 and position 2423 of Figure 6). A larger deletion, leaving 1.0 kb upstream of the fusion point (pNS3b), expressed ß-galactosidase at a high level in the presence of NaCl but also at a low level in the absence of NaCl, whereas in the original clone ß-galactosidase was not expressed in the absence of NaCl. Two other deletion constructs, carrying 540 bp (pNS3e) or 440 bp (pNS3f) fragments, showed a low level or no expression with NaCl, respectively. This indicates that the DNA region involved in NaCl-dependent gene expression is located between 1000 and 440 bp upstream of the fusion point (see Figure 5).

**1.3.1 Deletion mapping of NS3**

To localize sequences essential for salt-induced gene expression, a number of deletion derivatives of the pORI13 derivative recovered from the chromosome of NS3 (pNS3) were constructed. pNS3 was digested with *Hin*dIII yielding a fragment carrying *lacZ* and 1.0 kb of upstream lactococcal DNA. This fragment was ligated to a *Hin*dIII fragment of pORI13 carrying the Em resistance marker and the plus origin of replication of pWV01. The resulting construct, pNS3b, was isolated from *E. coli* EC1000. pNS3d was made by self-ligation of *Pst*I digested pNS3 and carries 2.4 kb of chromosomal DNA fused to *lacZ*. The proper construct was obtained in *L. lactis* LL108. Plasmid pNS3e was obtained by using the self-ligation mixture of an EcoRI digest of pNS3 to transform E. coli EC1000 and carries an about 540-bp chromosomal DNA fragment upstream of lacZ. pNS3f was constructed by ligating the XbaI fragment from pNS3III (see 1.4.1 below) to the XbaI site of pORI13 and transformation of *E. coli* EC1000. pNS3f carries an about 440 bp chromosomal DNA fragment upstream of lacZ (see Figure 5).

**1.4 Sequence analysis of the NS3 genomic region**

The 2.4 kb *Pst*I-*Sau*3A fragment, as present in pNS3d, was sequenced (Figure 6, which also gives the nucleotide sequence of the 561 bp *Sau*3A-*Xba*I fragment described in 1.4.1 below). This revealed a truncated open reading frame, *orfX*, that had been fused to *lacZ*. *OrfX* started about 360 bp upstream of the *Sau*3A site used for cloning and its putative truncated product shows homology with membrane proteins. The most extensive homology was found with two *E. coli* proteins with unknown function, YJEM and YGJI (Genbank entries U14003, and U18997, respectively). *OrfX* is preceded by a ribosome binding site (RBS). Upstream of *orfX* an ORF with a weak RBS could encode a protein of 276 amino acid residues (see SEQ. ID. NO: 10 and 11) with a calculated molecular weight of 32990. It shows homology to the *Streptococcus gordonii rgg* gene product (see SEQ. ID. NO: 12), which regulates expression of the glucosyl transferase gene (see ref. 27; M.C. Sulavik c.s.; 1992) and that of a partially sequenced ORF downstream of the *L. lactis pip* gene (see Figure 7, SEQ.ID. NO: 13 and ref. 30; B.L. Geller c.s; 1993). In analogy to the *S. gordonii* gene **the putative lactococcal regulator gene was named *rggL***. The overall identity between *rggL* and *rgg* is 24.3%, an additional 15.9% of the residues is similar. Directly downstream of *rggL* a 21 bp inverted repeat structure with a ΔG[25°C] of -37.8 kcal/mol followed by a stretch of T residues might function as a rho-independent terminator. Upstream of *rggL* a third, incomplete, ORF is present that shows homology to several *rnhB* genes, encoding RNAse HII (Figure 8 and SEQ. ID. NO: 14-17). The *rnhB* gene is followed by a rho-independent terminator-like structure with a ΔG[25°C] of -10.6 kcal/mol suggesting that the *rnhB* gene is transcribed independently from *rggL*.

The genomic organization of the NS3 locus is given in Figure 9, which shows that two rare restriction enzyme sites are present on the sequenced fragment, one for *Not*I (nucleotides 306-313, Figure 6) and one for *Apa*I (nucleotides 2382-2387, Figure 6), separated by only about 2070 bp. From a comparison with the genetic map of *L. lactis* MG1363 (see ref. 32; P. Le

Bourgeois c.s.; 1995) it is clear that the NS3 locus is positioned between the *ldh* gene and the *leu-ilv* gene cluster.

**1.4.1 Sequencing of the NS3 locus**

The 2.5 kb region upstream of the *lacZ*-fusion point in the chromosome of *L. lactis* NS3 was recovered into several subclones and sequenced. A 401 bp *Eco*RI-*Hin*dIII fragment from pNS3b was cloned in pUC19 and designated pNS3bI. 784 bp and 470 bp *Xba*I fragments from pNS3 were cloned in the *Xba*I site of pUC18, resulting in pNS3II and pNS3III, respectively. A 604 bp *Hin*dIII fragment from pNS3 was cloned in pUC18 and the plasmid was designated pNS3IV and a pUC18 derivative carrying the 743 bp *Eco*RI-*Xba*I fragment from pNS3 was called pNS3V. A fragment of 745 bp liberated from pNS3 with *Hin*dIII and *Pst*I and cloned in pUC19 resulted in pNS3VI. *E. coli* NM522 was used as a cloning host for pUC18 or pUC19 constructs. A fragment located at the 3'-end of the chromosomal insert in pNS3 was amplified by inverse-PCR. Self-ligated circularized fragments of a chromosomal *Xba*I digest were used as a template and NS3-5 and NS3-11 were used as primers. A 561 bp *Sau*3A-*Xba*I fragment was isolated from the PCR fragment and ligated into *Xba*I-*Bam*HI digested pORI19, resulting in pNS3i4 (see Figure 27). DNA sequencing was done on double-stranded plasmid DNA by the dideoxy chain-termination method (see ref. 4; F. Sanger c.s.; 1977) and the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the manufacturer's instructions. The double stranded sequence was completed by using synthetic oligonucleotide primers NS3-1, NS3-2, NS3-6, NS3-9, and NS3-10 (see Table 2 above and Figure 10).

**1.5 Identification of a salt-inducible promoter structure in NS3**

On the basis of the deletion studies and the nucleotide sequence, an NaCl-dependent start point of transcription was expected in the 100 bp region between the inverted repeat and the start codon of *orfX*. A primer extension was carried out on RNA isolated from LL108(pNS3d) grown in the absence and presence of 0.5 M NaCl. Using primer NS3-11 transcription was found to start at an A residue 68 bp upstream of the AUG start codon of *orfX* (position 2001, Figure 6). No primer extension product was obtained with RNA of cells grown in the absence of NaCl. 9 bp upstream of the transcription start site a -10 hexanucleotide was identified that differs in only one nucleotide from the consensus sequence (see ref. 25; M. van de Guchte c.s.; 1992). Further upstream no -35 like hexanucleotide could be discerned. Instead, 17 bp upstream of the -10 hexanucleotide the inverted repeat is located, suggesting a role for this structure in gene expression.

**1.5.1 RNA analysis**

RNA was isolated from exponentially growing *L. lactis* cultures (optical density at 600 nm of 0.5) with or without 0.5 M NaCl as previously described (see ref. 29; M. van Asseldonk; 1993). Northern hybridizations were done at 40°C in a buffer containing 50% formamide, 7% SDS, 2% blocking reagent (Boehringer, Mannheim, Germany), 5 x SSC, 50 mM sodium phosphate pH 7, and 0.1% N-lauryl sarcosine. A 470 bp *Xba*I fragment of pNS3III was used as a probe and labeled with [$\alpha^{32}$P-dCTP]. A synthetic oligonucleotide (NS3-11) complementary to the mRNA (position +96 to +133 downstream of the transcription start point) was used for primer extension. Twenty five nanogram of primer were added to 5 $\mu$g of RNA in a reaction mixture containing dCTP, dGTP, dTTP and $\alpha$-$^{35}$S-dATP and cDNA was synthesized using AMV reverse transcriptase (Boehringer, Mannheim, Germany). After 10 minutes incubation at 42°C an excess cold dATP was added and incubation was prolonged for another 10 minutes at 42°C. The product was analyzed on a sequencing gel next to a sequence reaction with the same primer, providing a size marker.

**1.6 RggL functions as an activator**

The involvement of *rggL* in NaCl-induced gene expression was examined using the *rggL* insertion mutant MGNS3i3 (see 1.6.1 below). RNA was isolated from MG1363 and MGNS3i3 cultured with and without NaCl and transferred to a filter. The 440 bp

*Xba*I-*Sau*3A fragment carrying the 5'-end of *orfX* was used as a probe in Northern hybridization. A strong hybridization signal was seen with RNA from MG1363 grown with NaCl (see Figure 11). Only a very weak signal was detected in the absence of NaCl, confirming the results obtained with the *lacZ* fusions. The same weak signal was seen with RNA from the *rggL* insertion mutant, both from cells grown with and without NaCl. This indicates that RggL acts as a positive regulator in NaCl-dependent expression of *orfX*. The size of the NaCl-dependent transcript in MG1363 was estimated to be approximately 3.0 kb.

**1.6.1 Construction of an *rggL* insertion mutant**

An internal *Xba*I-*Hin*dIII fragment of the *rggL* gene was cloned in the integration vector pORI19S (see Figure 28), using the $RepA^+$ *E. coli* helper strain EC101. This plasmid, pNS3i3, was used to disrupt the *rggL* gene in *L. lactis* MG1363, as described before (see ref. 37; J. Law c.s.; 1995). The proper chromosomal location of the integrated plasmid was confirmed by Southern hybridization (not shown) and the strain was named MGNS3i3.

<u>**Example 2**</u>

***lacZ* expression from NS3 is halide-ion-dependent**

In agar plates a number of compounds were tested to reveal the nature of the inducing condition (see Table 3 below). *lacZ* expression appeared to be independent of the osmolarity of the medium but was strictly linked to the presence of $Cl^-$ or $I^-$. ß-galactosidase activity increased with increasing NaCl concentrations in the medium (Figure 12). No induction of ß-galactosidase activity was observed by increasing the growth temperature.

<u>**Example 3**</u>

**Salt-induced expression of phage r1-t *lytPR* genes prevents outgrowth of a lactococcal culture**

The transcription start point upstream of *orfX* and *rggL* were amplified as a cassette using primer NS3-7 and NS3-8 and cloned upstream of the holin and lysin genes (*lytPR*) of the lactococcal temperate bacteriophage r1-t (see ref. 38; D. van Sinderen c.s.; 1996 and ref. 35; Quest International B.V. (A. Nauta c.s.); WO 95/31562). The start codon of *orfX* was fused

TABLE 3. *lacZ* expression in strain NS3 in the presence of different compounds

| Compound[a] | concentration (Mol/l) | ß-galactosidase activity[b] |
|---|---|---|
| none | - | - |
| Maltose | 0.5 | - |
| Mannitol | 0.5 | - |
| Sorbitol | 0.41 | - |
| Sucrose | 0.5 | - |
| $K_2SO_4$ | 0.25 | - |
| $MgSO_4$ | 0.25 | - |
| NaAcetate | 0.25 | - |
| $Na_2SO_4$ | 0.15 | - |
| $Na_2$Succinate | 0.25 | - |
| $NH_4$Acetate | 0.25 | - |
| $CaCl_2$ | 0.05 | + |
| KCl | 0.3 | + |
| KI | 0.3 | + |
| $MgCl_2$ | 0.125 | + |
| NaI | 0.3 | + |
| $NH_4Cl$ | 0.25 | + |

[a] Chemicals were added at the indicated final concentrations to GM17 agar containing Em and X-gal
[b] - : white colonies
+ : blue colonies

in-frame to ORF5 of r1-t which stops 13 bp upstream of the start codon of *lytP*, resulting in efficient translation of *lytP* and *lytR* (Figure 13A and SEQ. ID. NO: 18). Cultures of the resulting strain, LL108(pNS3PR), were grown in GM17 and induced at an optical density at 600 nm (OD600) of 0.5 by the addition of 0.5 M NaCl. Figure 14, lane 4 shows that 6 hours after induction, in addition to wild-type autolysin activity, phage lysin activity was present in the induced cells, and absent in the cells grown without NaCl. The addition of NaCl led to a stop in the increase of the OD600 of LL108(pNS3PR) followed by a decrease in optical density 4 hours after induction (Figure 15). The control strain, LL108(pNS378) expressing *lacZ*, continued to grow after NaCl addition, albeit at a lower rate.

**3.1 Construction of a transcriptional fusion of the NS3 promoter with the phage r1-t holin and lysin genes**

A 1280 bp fragment, encoding *rggL* and the salt-inducible promoter, was amplified by PCR using primers NS3-7 and NS3-8 (see Table 2 above) and pNS3 as template. This fragment was digested with *Sac*I and *Eco*RV and ligated to pIR1PR (see ref. 35; Quest International B.V. (A. Nauta c.s.); WO 95/31562) linearized with *Sac*I and *Sca*I. The ligation mixture was used to transform *L. lactis* LL108 and the resulting plasmid was called pNS3PR (see Figure 16). As a negative control for lytic activity, the same PCR fragment was cloned upstream of *lacZ* in pORI13. The PCR fragment was cut with *Bgl*II and *Eco*RV and ligated to *Bgl*II-*Sma*I-digested pORI13. The ligation mixture was used to transform LL108 and the resulting plasmid was labeled pNS378.

<u>Example 4</u>

**Salt-induced expression of the lactococcal *acmA* gene results in the stabilization of the OD600 of a culture**

The cassette containing *rggL*, the salt-inducible promoter, and the RBS and start codon of *orfX* was placed upstream of *acmA*, the gene of the major peptidoglycan hydrolase of *Lactococcus lactis* (see ref. 34; Quest International B.V. (G. Buist c.s.); WO 95/31561 and ref. 31; G. Buist c.s.; 1995). Two mutations occurred in the fusion region. An A to G transition in the untranslated leader of the transcript (see nucleotide 64 in Figure 13A + 13B and SEQ. ID. NO: 18 and SEQ. ID. NO: 19) and a deletion of an A residue (see nucleotide 99 in Figure 13B and SEQ. ID. NO: 19) resulting in the loss of the *Sca*I site at the fusion point. Translation of the fusion transcript results in the formation of a heptapeptide from the RBS of *orfX* and the translation of *acmA* from its own

RBS (Figure 13B and SEQ. ID. NO: 19). Translation of the small peptide stops 10 bp upstream of the RBS of *acmA*. The growth of the strain carrying the resulting plasmid, pNS3AL3, did not differ from the control strain in GM17 broth (Figure 17). However, upon addition of NaCl the cells grew slower and the OD600 stabilized after 4 hours. The OD600 of the control strain increased to a higher level in the presence of salt. More AcmA activity was detected in cells induced with NaCl compared to uninduced cells and to control cells expressing ß-galactosidase under control of the same induction cassette (Figure 14). Clearly, the expression of *acmA* from pNS3AL3 is induced by NaCl.

**4.1 Construction of a transcriptional fusion of the NS3 promoter with the lactococcal autolysin gene *acmA***

The *acmA* gene interrupted by a *Sac*I fragment on pAL10 was used as a basis (see ref. 34; Quest International B.V. (G. Buist c.s.); WO 95/31561). The *Bgl*II sites in pAL10 were deleted by cutting with *Bgl*II, filling the overhanging ends with Klenow polymerase, recircularization with T4 ligase and transformation of EC1000. This construct, pAL101, was linearized with *Bam*HI and *Xba*I and ligated to pORI28, also linearized with *Bam*HI and *Xba*I. The proper construct, pAL102, was isolated from *E. coli* EC1000. A PCR fragment, made by amplification using NS3-7 and NS3-8 on pNS3 as the template and described in Example 3.1 above, was cut with *Bgl*II and *Sca*I and ligated to pAL102, linearized with the same restriction enzymes. The ligation mixture was used to transform *L. lactis* LL302 and pNS3AL3S was recovered. This plasmid was digested with *Sac*I to delete the insert in *acmA*. After self-ligation the mixture was used to transform LL302. The resulting construct, carrying an intact *acmA* copy fused to the NS3 salt-inducible promoter, was designated pNS3AL3 (see Figure 18).

__Example 5__

**Intracellular proteins are released upon induction of lysin genes**

The supernatant of the holin-lysin expressing strain (see ref. 35; Quest International B.V. (A. Nauta c.s.); WO 95/31562) contains a significant amount of cytoplasmic proteins 6 hours after NaCl addition, whereas in the supernatant of the same strain grown without induction only secreted proteins are visible (Figure 19.A, lane 3). The addition of NaCl to the control strain already causes the release of a small portion of cytoplasmic proteins (Figure 19.A, lane 2). In the supernatant of cells overexpressing AcmA a much larger quantity of cytoplasmic proteins was observed (Figure 19.A, lane 6) when compared to the amount of intracellular proteins released from LL108(pNS3PR). To detect lytic activity, sodium dodecyl sulphate (12.5%) polyacrylamide gel-electrophoresis was carried out as described (see ref. 1; U.K. Laemmli; 1970) whereby 0.2% autoclaved, lyophilized *Micrococcus lysodeikticus* cells were included in the gel (see ref. 31; G. Buist c.s.; 1995). In Figure 19.B, lane 4 it was shown that LytR activity was detectable in the supernatant of induced LL108(pNS3PR). Using the same method it was also shown that a high level of AcmA activity was present in the supernatant of induced LL108(pNS3AL3). A number of breakdown products of AcmA were visible, as well as the precursor form of AcmA, which is normally present in the cytoplasm (see ref. 34; Quest International B.V. (G. Buist c.s.); WO 95/31561 and ref. 31; G. Buist c.s.; 1995). This latter protein is not processed by the protein export machinery and, therefore, must have been released by cell lysis. AcmA activity in the NaCl-induced samples of LL108(pNS378) and LL108(pNS3PR) was lower than in non-induced samples. This is, most likely, due to the lower OD of the corresponding culture at the moment of sampling (see Figure 15 and Figure 17).

### 5.1 Determination of peptidase activity in the supernatant of *L. lactis* cultures

In order to quantify the release of intracellular proteins PepXP was chosen as an intracellular marker enzyme. Cell lysis was quantified by measuring PepXP release from cells lacking the chromosomal autolysin gene. MG1363*acmA*Δ1-(pVE6007) was transformed with either pNS3PR or pNS3AL3. PepXP activity in supernatant samples was determined by following hydrolysis of the synthetic substrate Ala-Pro-*p* nitroanilide at 405 nm for 20 minutes at 37°C in a 96-well microtiterplate using a Thermomax microplate reader (Molecular Devices Co., Menlo Park, CA). The presence of protoplasts in induced cultures was tested by washing 1 ml of cells in medium containing a concentration of NaCl which was identical to that in the original culture. Subsequently, half of the cells were resuspended in 0.5 ml of (hypoosmotic) M17 and the other half in 0.5 ml M17 containing the original NaCl concentration. Samples were incubated for 30 minutes at 37°C and PepXP activity in the supernatants was measured. PepXP activity in the hypoosmotic extractable cell fraction is given as the activity in the first 0.5 ml corrected for the activity in the latter 0.5 ml.

In order to exclusively study the effect of the salt-induced lysis genes PepXP activity in culture supernatants was followed after induction of either lysin gene with 0.5 M NaCl at a culture OD600 of 0.5. During the first hours after induction low levels of PepXP were detected in the culture supernatants (Figure 20). Optimal PepXP levels were obtained 8 hours after induction of cultures of MG1363*acmA*Δ1(pVE6007)-(pNS3AL3) and 30 hours after induction of MG1363acmAΔ1-(pVE6007)(pNS3PR). The highest level of PepXP activity was present after induction with 0.1 M NaCl (Figure 21). Highest PepXP levels were reached at 30 hours after induction with 0.1 M NaCl of strains carrying the NS3::*lytPR* fusion and 70 hours after induction of the NS3::*acmA* strain (Figure 20). The control strain, carrying NS3::*lacZ*, released low levels of PepXP activity after prolonged incubation. PepXP activity

is stable for at least 40 hours in a cell extract in M17, either with or without NaCl (data not shown).
The addition of NaCl to a culture not only induces the activity of the NS3 promoter but also increases the osmolarity of the medium. This probably results in stabilization of osmotically fragile cells formed by lysin activity in the cultures. By incubation in hypoosmotic medium extra PepXP activity could be released from cells carrying the NS3::*lytPR* fusion induced with 0.1, 0.25, or 0.5 M NaCl, indicative of the formation of fragile cells by the action of LytPR (Figure 21). Induction of *acmA* expression with NaCl resulted in much lower levels of PepXP in the culture medium compared to induction of *lytPR*. However, much more PepXP was extractable with hypoosmotic medium from NS3::*acmA* carrying cells. The sum of PepXP in the supernatant and PepXP extractable from cells is comparable for NS3::acmA and NS3::lytPR cells induced with 0.25 M NaCl. The amount of PepXP extracted from NS3::acmA cells increased with the amount of NaCl used for induction. Only after induction with 0.5 M NaCl a smaller amount of PepXP was extracted. Cells that constitutively overexpress AcmA {MG1363(pGKAL1)} released slightly more PepXP in the presence of 0.1 M NaCl as compared to M17 without added NaCl. At higher NaCl concentrations less PepXP was liberated from these cells. Also from this strain, incubated in 0.25 or 0.5 M NaCl, high levels of PepXP were extractable with hypoosmotic M17. Apparently, AcmA activity results in a weaker cell wall but not in cell lysis in medium containing NaCl.
KCl could replace NaCl as the inducing agent, resulting in only slightly lower levels of released PepXP (data not shown).

**Example 6**

**A chloride and low pH-inducible acid resistance mechanism in *Lactococcus lactis*.**

In this Example the term *gad* is used to indicate a group of genes involved in glutamate-dependent acid resistance. **Therefore, the genes indicated above with *rggL* and *orfX* have**

**now been renamed as *gadR* and *gadC*, respectively.** The reason for the abbreviation "rggL" was discussed in Example 1.4 above. The reason for the abbreviations "gadB" "gadC" "gadR" is discussed below under the heading RESULTS.

**Additional MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.**

The **(additional)** bacterial strains and plasmids used in this Example are listed in Table 4. *L. lactis* was grown at 30°C in half strength M17 (½M17) broth containing 0.5% glucose and 1.9% ß-glycerophosphate; solidified ½M17 contained 1.5% agar. Modified M17 (mM17) contained no ß-glycerophosphate and no soytone. ß-glycerophosphate was added to a final concentration of 2%, where indicated.

**General DNA techniques.**

Protein homology searches against the Genbank were carried out using the BLAST program (Altschul *et al.*, 1990; ref. 41). Protein sequence alignments were carried out with the PALIGN program of PC/Gene using the structure genetic matrix. Transmembrane segments were predicted using the method of Klein *et al.* (1985; ref. 45)

**Cloning of *gadCB* of *L. lactis***

The region downstream of the *lacZ* fusion site in *L. lactis* NS3 was obtained by inverse PCR amplification of a 0.56-kb *Sau*3A-*Xba*I fragment from *L. lactis* MG1363 chromosomal DNA using primers NS3-5 and NS3-11 (see Table 2 above). The PCR product was cloned in pORI19 using *Escherichia coli* EC101 as a host, **resulting in pNS3i4** (see Example 1.4.1 above and Figure 27). In a subsequent inverse PCR the adjoining 0.9-kb *Xba*I-*Sau*3A chromosomal DNA fragment was amplified using primers NS3-14 (5' GGCAGTCCGTTGCGTCCCACC) and NS3-15 (5' GAGTTATCATTAAGTGCAGGGG) and cloned in pORI19 using *L. lactis* LL108 as the cloning host. This construct was named pNS3i5 (see Figure 27) and used for single cross-over integration, **resulting in strain MGNS3i5.** Chromosomal DNA of MGNS3i5 was digested with *Eco*RI to clone sequences downstream of the insertion site of pNS3i5. The chromosomal *Eco*RI fragments were circularized by self-ligation and the ligation mixture was used to transform *L. lactis* LL108. Transformants contained a 4.8-kb plasmid (pNS3i6; see Figure 27) **which is pORI19** containing a 2.6-kb *Xba*I-*Eco*RI fragment from the chromosome (Figure 22).

Table 4. Additional bacterial strains and plasmids for Example 6.

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| *L. lactis* | | |
| LL302 | MG1363 derivative, carrying a single *repA* copy in *pepXP* | Leenhouts, unpublished, See earlier Examples |
| MGNS3i3 | Sp$^r$, derivative of MG1363 with pNS3i3 inserted in *gadR* | See earlier Examples |
| MGNS3i4 | Em$^r$, derivative of MG1363 with pNS3i4 inserted in *gadC* | This work |
| MGNS3i5 | Em$^r$, MG1363 derivative carrying a single copy of pNS3i5 on the chromosome | This work |
| MGNS3i7 | Em$^r$, derivative of MG1363 with pNS3i7 inserted in *gadB* | This work |
| MGNS31 | Em$^r$, MG1363 with *lacZ* inserted between *gadR* and $P_{gad}$ | This work |
| MGNS32 | Em$^r$, MG1363 (*gadR*::*lacZ*) | This work |
| *E. coli* | | |
| EC101 | Km$^r$, JM101 derivative, carrying a single copy of the pWV01 *repA* gene in *glgB* | Law *et al.*, 1995; ref. 37 |
| Plasmids | | |
| pORI13 | Promoterless *lacZ*, Em$^r$, Ori$^+$, RepA$^-$ derivative of pWV01 | See earlier Examples |
| pNS3i3 | Sp$^r$, pORI19S carrying a 240-bp *Xba*I-*Hin*dIII fragment internal to *gadR* | See earlier Examples |
| pNS3i5 | Em$^r$, pORI19 carrying a 0.9-kb *Xba*I-*Sau*3A chromosomal DNA fragment encoding the 3'-end of *gadC* and the 5'-end of *gadB* | This work |
| pNS3i6 | Em$^r$, pORI19 carrying a 2.4-kb *Xba*I-*Eco*RI chromosomal DNA fragment | This work |
| pNS3i7 | Em$^r$, pORI19 carrying a 500-bp internal *Ase*I fragment of *gadB* | This work |

**Construction of insertion mutants.**

An internal *Xba*I-*Hin*dIII fragment of *gadR* was cloned as a blunt fragment in the *Sma*I site of pORI19S (see Figure 28) using the RepA$^+$ *E. coli* helper strain EC101. **The resulting plasmid, pNS3i3**, was used to disrupt *gadR* in *L. lactis* MG1363, as described before (Law *et al.*, 1995; ref. 37). The proper chromosomal location of the integrated plasmid was confirmed by Southern hybridization and **the strain was named MGNS3i3. Plasmid pNS3i7 was constructed** by cloning a 0.5-kb *Asn*I fragment (internal to *gadB*) as a blunt fragment in the *Sma*I site of pORI19 using *E. coli* EC101 as a host (see Figure 27). **Single cross-over integration of pNS3i4 and pNS3i7 in the *L. lactis* MG1363 chromosome resulted in strains MGNS3i4 (*gadC*) and MGNS3i7 (*gadB*), respectively.**

**ß-Galactosidase assays.**
Cell-free extracts were prepared by vigorous shaking of cells in the presence of glass beads (van de Guchte *et al.*,1991; ref. 24). ß-Galactosidase activity was determined as described by Miller (1972; ref. 2). Protein concentrations were determined by the method of Bradford (1976; ref. 3) with bovine serum albumin as the standard.

**Acid resistance tests.**
Cells of an exponentially growing culture of *L. lactis* in half strength GM17 (½GM17) with or without 0.3 M NaCl was harvested, washed with water and resuspended in an equal volume of MS15 (Cocaign-Bousquet *et al.*, 1995; ref. 42) without glucose and glutamate and containing the same amount of NaCl as the culture. The pH of MS15 was adjusted to 3.5 with either lactic acid or hydrochloric acid prior to cell resuspending. After incubation for 2 hours at 30°C the number of viable cells was determined by plating onto glucose ½M17 plates. The percentage of acid resistant cells was calculated from the number of colony-forming units (cfu) after acid treatment divided by the number of cfu found at the moment of harvest.

**RESULTS**

**Nucleotide sequence of the genes transcribed by the chloride-dependent promoter.**

In the previous Examples the chloride-dependent promoter ($P_{gad}$) was identified using a random *lacZ* chromosomal integration strategy. Here the nucleotide sequence is presented of the chromosomal region downstream of the *lacZ* integration site in *L. lactis* NS3.

The original *Sau*3A fusion site in the *lacZ* integrant *L. lactis* NS3 is located in an ORF of 503 codons immediately downstream of $P_{gad}$. This ORF was named *gadC*, as its deduced amino acid sequence (Figure 23) is homologous to GadC from *Shigella flexneri* (51% identity and 17% similarity, Waterman and Small, 1996; ref. 47) and its *E. coli* counterpart XasA (Hersh *et al.*, 1996; ref. 43). GadC is homologous to a number of amino acid antiporters, including the lysine-cadaverine antiporter CadB from *E. coli* (Meng and Bennet, 1992; ref. 46). Lactococcal GadC has a deduced molecular weight of 55369 and a pI of 9.73 and is highly hydrophobic. The hydrophobic residues are clustered in 12 domains (Figure 23), whose locations coincide with those of the hydrophobic domains in *S. flexneri* GadC (as predicted by a number of topology-predicting computer programs). This suggests that GadC is an integral membrane protein. A conserved domain found in glutamate transporting proteins is also present in *L. lactis* GadC (Figure 23, Waterman and Small, 1996; ref. 47); *gadC* is separated by 19-bp from **another ORF of which the deduced protein is homologous to glutamate decarboxylases**. Highest homology is found with glutamate decarboxylase from *Synechocystis* sp. (48% identity and 15% similarity, Figure 24, Kaneko *et al.*, 1996; ref. 44). Both in *E. coli* and *S. flexneri gadB* is linked to the putative glutamate - γ-amino butyrate antiporter genes, *xasA* and *gadC*, respectively. **The lactococcal gene was**, in analogy to *E. coli*, and *S. flexneri* **named *gadB***. The gene order of *gadB* and *gadC* is inverse in *L. lactis* compared to these organisms. No possible transcription signals could be identified in the 19-bp intergenic region between *gadC* and *gadB* which suggests that they form an operon. Downstream of *gadB* a 16-bp inverted repeat (IR) may function as a rho-independent transcription terminator.

**_gadR_ is constitutively expressed**

Expression of *gadR* was studied in strain MGNS32 carrying a single copy *gadR::lacZ* transcriptional fusion (see Figure 22). ß-galactosidase activity in exponentially growing MGNS32 was 3.0 U/mg, independent of the presence of NaCl. In other words, NaCl-dependent expression from $P_{gad}$ is not regulated by variations in the level of transcription of *gadR*. Another *lacZ* fusion, located immediately downstream of the 21-bp IR was used to show that transcription of *gadR* is effectively terminated by this IR. No ß-galactosidase activity could be detected in strain MGNS31 carrying this fusion (see Figure 22). Transcription of *gadR* starts from a C-residue 116 bp upstream of the AUG start codon (Figure 22). The *gadR* promoter consists of canonical -35 and -10 hexanucleotides separated by 18 bp (Figures 6 and 29).

**Expression of _gadCB_ is enhanced at low pH and by glutamate**

In *S. flexneri* and *E. coli* *gadC* and *xasA* have been shown to play a role in survival at low pH in the presence of glutamate (Waterman and Small, 1996; ref. 47; Hersh *et al.*, 1996; ref. 43). The expression of lactococcal *gadC* under such conditions was studied in *L. lactis* NS3. Strain NS3 was previously identified because of its chloride-dependent *lacZ* expression and contains a single copy *gadC::lacZ* fusion on its chromosome (see previous Examples). A modified M17 medium (mM17) was used, lacking the buffer ß-glycerophosphate to modulate the culture pH; mM17 also lacks soytone. ß-galactosidase activity in NS3 was still induced in this modified medium in the presence of 0.3 M NaCl and 2% ß-glycerophosphate buffer but the induction level was fivefold lower as compared to that in standard ½M17 (data not shown). The *lacZ* expression in mM17 in the presence of NaCl was low in the early stages of exponential growth and increased to an optimum at the onset of the stationary phase (Figure 25). In the absence of buffer, the culture pH decreased to 4.0 to 4.5

in the stationary growth-phase, while cultures containing buffer reached a lowest pH of 5.5. Expression of *lacZ* was increased 10-fold in mM17 containing no buffer. The presence of 50 mM glutamate resulted in an approximately twofold additional increase in the expression of *lacZ* both in buffered and in unbuffered mM17 broth (Figure 25). No ß-galactosidase activity was detectable in the absence of NaCl whether or not glutamate was present and independent of the culture pH (data not shown). Therefore, expression of *gadCB* is optimal at the onset of the stationary growth phase in the presence of NaCl and glutamate, and at low pH.

***gadCB* confers acid resistance**

The ability of *L. lactis* MG1363 to survive acid stress was tested under conditions where *gadCB* is expressed and under conditions where *gadCB* is not expressed. When MG1363 was grown in ½GM17 and acid-challenged at pH 3.5 in MS15 for two hours the viability of the cells decreased dramatically (Table 5).

Table 5. Acid resistance of *L. lactis* MG1363 under different conditions of challenge

| conditions: | % acid resistance at pH 3.5, lactic acid | % acid resistance at pH 3.5, HCl | % acid resistance at pH 6.5 |
|---|---|---|---|
| - | 0.0005 | 1.12 | 139 |
| + 1 mM glutamate | 0.0004 | 0.96 | 135 |
| + 0.3 M NaCl | 0.0002 | 1.00 | 58 |
| + 0.3 M NaCl<br>+ 1 mM glutamate | 0.49 | 16 | 57 |

Lactic acid was much more deleterious than hydrochloric acid whereas the viability was not affected at pH 6.5. The presence of 1 mM glutamate alone did not affect the viability at pH 3.5. In the presence of 0.3 M NaCl during growth, allowing *gadCB* expression, followed by acid challenge in the presence of 0.3 M NaCl and 1 mM glutamate the viability was reduced only 200-fold when lactic acid was used. Under the latter conditions, the viability in MS15 adjusted to pH 3.5 with hydrochloric acid was also enhanced significantly. Under conditions of *gadCB* expression (by the presence of 0.3 M NaCl) acid resistance was lower in the absence of glutamate than when glutamate was present (Table 5), indicating that glutamate is not only involved in induction of *gadCB* expression but is directly needed to confer acid resistance. To confirm the direct involvement of *gadCB* in acid resistance *L. lactis* MGNS3i3 (which does not express *gadCB*, see earlier Examples) was subjected to acid challenge.

Table 6. Acid resistance of *L. lactis gadCB* mutants.

| Strain | Genotype; | % acid resistance[a] in MS15 | % acid resistance[a] in MS15 + NaCl + glu |
|---|---|---|---|
| MG1363 | w.t. | 0.0005 | 0.49 |
| MGNS3i3 | *gadR* | 0.0003 | 0.0013 |
| MGNS3i4 | *gadC* ($GadB^-$)[b] | 0.00007 | 0.00009 |
| MGNS3i5 | ($GadB^-$)[b] | 0.00003 | 0.00026 |
| MGNS3i7 | *gadB* | 0.00001 | 0.00008 |

[a] at pH 3.5, lactic acid

[b] Insertions in *gadC* and in between *gadC* and *gadB* will, most likely, have a polar effect on *gadB*.

Survival in the presence of NaCl and glutamate was reduced to levels comparable to that of the wild-type strain in the absence of NaCl and glutamate (Table 6). An insertion mutant in *gadC* (strain MGNS3i4) showed a similar phenotype. This mutation, most likely, has a polar effect on *gadB* which would result in the absence of both GadC and GadB. An insertion mutant in *gadB* (strain MGNS3i7) is similarly acid sensitive, indicating a central role for GadB in the acid resistance process. Strain MGNS3i5, carrying intact copies of *gadC* and *gadB* separated by an integrated copy of pORI19 is acid sensitive, suggesting that *gadB* expression is abolished. These data show that *gadCB*, which is transcribed from the chloride-dependent *gad* promoter $P_{gad}$, is involved in glutamate-dependent acid resistance, as depicted in Figure 26.

**SUMMARY**

- *Lactococcus lactis* MG1363 has a glutamate-dependent acid resistance mechanism, that is active in the presence of chloride.
- *L. lactis* has two genes, *gadC* and *gadB*, that encode proteins homologous to a putative glutamate - γ-aminobutyrate antiporter and a glutamate decarboxylase, respectively, from Escherichia coli and *Shigella* flexneri. These genes are involved in glutamate-dependent acid resistance in *E. coli* and *S. flexneri*.
- The expression of *gadCB* in *L. lactis* is induced by chloride and is optimal at low pH in the presence of glutamate.
- *L. lactis* insertion mutants with a disrupted *gadB* or that are unable to express both *gadB* and *gadC* are more sensitive to low pH than the wild-type when NaCl and glutamate are present, indicating that the lactococcal *gadCB* operon is involved in glutamate dependent acid resistance, see Figure 26.

**Conclusions to be drawn from Example 6**

The results of further research given in Example 6 have shown that this salt-inducible promoter is more active at a lower pH. For example, Figure 25A shows that upon induction with 0.3 M NaCl the yield of ß-galactosidase is not more than about 8 Units/mg when the medium is buffered such that the pH will not come below 5.5.

In contrast therewith, Figure 25B shows that in the absence of a buffer the pH can drop to about 4, while under these conditions the yield of ß-galactosidase is about 80 Units/mg.

The salt-inducible promoter is more active at a lower pH and has thus also become pH-inducible in the presence of salt. The activity of the salt- and pH-inducible promoter can be further enhanced in the presence of glutamate/glutamic acid, as is shown in Figure 25A and 25B. They show that production of ß-galactosidase is increased from 8 to 15 Units/mg in a buffered medium and from 80 to 225 Units/mg in a non-buffered medium when the pH during fermentation has dropped to about 4.3.
Although the scope of the invention is not limited by any theory, it is supposed that these enhancing effects are caused by control of the internal pH by the uptake of glutamate and decarboxylation whereby γ-aminobutyrate is formed and subsequently secreted under influence of the *gadB* and *gadC* genes described in this Example.

Thus Example 6 shows that a desired protein can be produced in a lactic acid bacterium at an improved yield by using a construct in which the gene encoding the desired protein is under control of a salt- and pH-inducible promoter and the medium in which the transformed lactic acid bacterium is cultured is not buffered and preferably contains glutamate-/glutamic acid. For an easy recovery it is generally desirable that the desired protein contains a secretion signal sequence to enable secretion of the protein.

Alternatively, if the transformed lactic acid bacterium also contains at least one gene encoding a lytic protein under control of the salt- and pH-inducible promoter, proteins without a secretion signal sequence can be easily recovered, because the lytic protein will perforate the cell wall so that the contents including the desired protein can be released from the cell.

**REFERENCES**

1. **U.K. Laemmli**; Nature (London) 227 (**1970**) 680-685; Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4

2. **J.H. Miller; (1972)**; Experiments in molecular genetics Cold Spring Harbor laboratory, Cold Spring Harbor New York p. 352-359; Assay of ß-Galactosidase Induction; Time Course of ß-Galactosidase Induction

3. **M.M. Bradford**; Anal. Biochem. 72 (**1976**) 248-254; A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding

4. **F. Sanger, S. Nicklen, and A.R. Coulson**; Proc. Natl. Acad. Sci. USA 74 (**1977**) 5463-5467; DNA sequencing with chain-terminating inhibitors

5. **M.J. Gasson**; J. Bacteriol. 154 (**1983**) 1-9; Plasmid - Complements of *Streptococcus lactis* NCDO712 and Other Lactic Streptococci after Protoplast-Induced Curing

6. **J.A. Gough, and N.E. Murray**; J. Mol. Biol. 166 (**1983**) 1-19; Sequence Diversity among Related Genes for Recognition of Specific Targets in DNA Molecules

7. **G.M. Weinstock, M.L. Berman, and T.J. Silhavy**; In: T.S. Papas, M. Rosenberg, and J.G. Chirikjian (eds), Gene amplification and analysis III. Elsevier, North Holland, New York, (**1983**) pp. 27-64; Chimeric genes with ß-galactosidase.

8. **C. Yanisch-Perron, J. Vieira, and J. Messing**; Gene 33 (**1985**) 103-119; Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors

9. **S.A. Lacks, P. Lopez, B. Greenberg, and M. Espinosa**; J. Mol. Biol. 192 (**1986**) 753-765; Identification and Analysis of Genes for Tetracycline Resistance and Replication Functions in the Broad-host-range Plasmid pLS1

10. **J.M.B.M. van der Vossen, D. van der Lelie, and G. Venema**; Appl. Environ. Microbiol. 53 (**1987**) 2452-2457; Isolation and Characterization of *Streptococcus cremoris* Wg2-Specific Promoters

11. **J.A.K.W. Kiel, J.P.M.J. Vossen, and G. Venema**; Mol. Gen. Genet. 207 (**1987**) 294-301; A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation

12. **W.R. Pearson and D. J. Lipman**; Proc. Natl. Acad. Sci. USA 85 (**1988**) 2444-2448; Improved tools for biological sequence comparison

13. **S.P. Chambers, S.E. Prior, D.A. Barstow, and N.P. Minton**; Gene **68** (**1988**) 139-149; The pMTL *nic*$^-$cloning vectors, I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing

14. **K.J. Leenhouts, J. Kok, and G. Venema**; Appl. Environ. Microbiol. 55 (**1989**) 394-400; Campbell-Like Integration of Heterologous Plasmid DNA into the Chromosome of *Lactococcus lactis* subsp. *lactis*.

15. **H. Holo, and I.F. Nes**; Appl. Environ. Microbiol. **55** (**1989**) 3119-3123; High-Frequency Transformation, by Electroporation, of *Lactococcus lactis* subsp. *cremoris* Grown with Glycine in Osmotically Stabilized Media

16. **J. Sambrook, E. F. Fritsch, and T. Maniatis**; Molecular cloning: a laboratory manual, 2nd ed. (**1989**); Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

17. **K.J. Leenhouts, J. Kok, and G. Venema**; Appl. Environ. Microbiol. 56 (**1990**) 2726-2735; Stability of Integrated Plasmids in the Chromosome of *Lactococcus lactis*

18. **M. Itaya**; Proc. Natl. Acad. Sci. USA 87 (**1990**) 8587-8591; Isolation and characterization of a second RNAse H (RNAse HII) of *Escherichia coli* K-12 encoded by the *rnhB* gene

19. **E.R. Zabarovsky and G. Winberg**; Nucleic Acids Res. 18 (**1990**) 5912; High efficiency electroporation of ligated DNA into bacteria

20. **B. Mayo, J. Kok, K. Venema, W. Bockelmann, M. Teuber, H. Reinke, and G. Venema**; Appl. Environ. Microbiol. 57 (**1991**) 38-44; Molecular Cloning and Sequence of an X-Prolyl Dipeptidyl Aminopeptidase Gene from *Lactococcus lactis* subsp. *cremoris*

21. **K.J. Leenhouts, J. Kok, and G. Venema**; J. Bacteriol. 173 (Aug. **1991**) 4794-4798; Replacement Recombination in *Lactococcus lactis*

22. **K.J. Leenhouts, J. Kok, and G. Venema**; Appl. Environ. Microbiol. 57 (Sept. **1991**) 2562-2567; Lactococcal Plasmid pWV01 as an Integration Vector for Lactococci

23. **J. Vieira and J. Messing**; Gene 100 (**1991**) 189-194; New pUC-derived cloning vectors with different selectable markers and DNA replication origins

24. **M. van de Guchte, J. Kok, and G. Venema**; Mol. Gen. Genet. 227 (**1991**) 65-71; Distance-dependent translational coupling and interference in *Lactococcus lactis*

25. **M. van de Guchte, J. Kok, and G. Venema**; FEMS Microbiol. Rev. 88 (**1992**) 73-92; Gene expression in *Lactococcus lactis*

26. **C.J. Leenhouts, J.D. Marugg and C.T. Verrips** (Unilever N.V./PLC); Patent Application EP-A1-0 487 159 published 27 May **1992**; A food-grade vector suitable for transforming a food-grade host cell, use of said vector for transforming food-grade host cells, and use of said transformed cells in biotransformation processes

27. **M.C. Sulavik, G. Tardif, and D.B. Clewell**; J. Bacteriol. 174 (**1992**) 3577-3586; Identification of a Gene, *rgg*, Which Regulates Expression of Glucosyltransferase and Influences the Spp Phenotype of *Streptococcus gordonii* Challis

28. **E. Maguin, P. Duwat, T. Hege, D. Ehrlich, and A. Gruss**; J. Bacteriol. 174 (**1992**) 5633-5638; New Thermosensitive Plasmid for Gram-Positive Bacteria

29. **M. van Asseldonk, A. Simons, H. Visser, W. M. de Vos, and G. Simons**; J. Bacteriol. 175 (**1993**) 1637-1644; Cloning, Nucleotide Sequence, and Regulatory Analysis of the *Lactococcus lactis dnaJ* Gene

30. **B.L. Geller, R.G. Ivey, J.E. Trempy, and B. Hettinger-Smith**; J. Bacteriol. 175 (**1993**) 5510-5519; Cloning of a Chromosomal Gene Required for Phage Infection of *Lactococcus lactis* subsp. *lactis* C2

31. **G. Buist, J. Kok, K.J. Leenhouts, M. Dabrowska, G. Venema, and A. Haandrikman**; J. Bacteriol. 177 (**1995**) 1554-1563; Molecular Cloning and Nucleotide Sequence of the Gene Encoding the Major Peptidoglycan Hydrolase of *Lactococcus lactis*, a Muramidase Needed for Cell Separation

32. **P. Le Bourgeois, M. Lautier, L. van den Berghe, M.J. Gasson, and P. Ritzenthaler**; J. Bacteriol. 177 (**1995**) 2840-2850; Physical and Genetic Map of the *Lactococcus lactis* subsp. *cremoris* MG1363 Chromosome: Comparison with That of *Lactococcus lactis* subsp. *lactis* IL1403 Reveals a Large Genome Inversion

33. **R.D. Fleischmann c.s.**; Science 269 (**1995**) 496-512; Whole-Genome Random Sequencing and Assembly of *Haemophilus influenzae* Rd

34. **G. Buist, G. Venema, J. Kok, and A.M. Ledeboer** (Quest International B.V.); International Patent Application WO-A1-95/31561 published 23 Nov. **1995** (PCT); PROCESS FOR THE LYSIS OF A CULTURE OF LACTIC ACID BACTERIA BY MEANS OF A LYSIN, AND USES OF THE RESULTING LYSED CULTURE

35. **A. Nauta, G. Venema, J. Kok, and A.M. Ledeboer** (Quest International B.V.); International Patent Application WO-A1-95/31562 published 23 Nov. **1995** (PCT); PROCESS FOR INHIBITING THE GROWTH OF A CULTURE OF LACTIC ACID BACTERIA, AND OPTIONALLY LYSING THE BACTERIAL CELLS, AND USES OF THE RESULTING LYSED CULTURE

36. **A. Nauta, G. Venema, J. Kok, and A.M. Ledeboer** (Quest International B.V.); International Patent Application WO-A1-95/31563 published 23 Nov. **1995** (PCT); COMPLEX INDUCIBLE PROMOTER SYSTEM DERIVABLE FROM A PHAGE OF A LACTIC ACID BACTERIUM (LAB), AND ITS USE IN A LAB FOR PRODUCTION OF A DESIRED PROTEIN

37. **J. Law, G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts**; J. Bacteriol. 177 (**1995**) 7011-7018; A System to Generate Chromosomal Mutations in *Lactococcus lactis* Which Allows Fast Analysis of Targeted Genes.

38. **D.van Sinderen, H. Karsens, J. Kok, P. Terpstra, M.H.J. Ruiters, G. Venema, and A. Nauta**; Mol. Microbiol. 19 (No.6) (**1996**) 1343-1355; Sequence analysis and molecular

characterization of the temperate lactococcal bacteriophage r1t

39. **K. Leenhouts, G. Buist, A. Bolhuis, A. ten Berge, J. Kiel, I. Mierau, M. Dabrowska, G. Venema, and J. Kok**; Mol. Gen. Genet. (1996, Accepted for publication); A general system for generating unlabelled gene replacements in bacterial chromosomes

= **K.J. Leenhouts, G. Buist, A. Bolhuis, A. ten Berge, J. Kiel, I. Mirau, M. Dabrowska, G. Venema, and J. Kok**; Mol. Gen. Genet. 253 (1996) 217-224; A general system for generating unlabeled gene replacements in bacterial chromosomes (Published after 5 Sep 1996)

40. **K.J. Leenhouts, A. Bolhuis, G. Venema, and J. Kok**; (Submitted for publication 1996); Construction of a food-grade multiple copy integration system for *Lactococcus lactis*.

41. **S.F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman**; J. Mol. Biol. 215 (1990) 403-410; Basic local alignment search tool

42. **M. Cocaign-Bousquet, C. Garrigues, L. Novak, N. D. Lindley, and P. Loubiere**; J. Appl. Bacteriol. 79 (1995) 108-116; Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactis*

43. **B.M. Hersh, F. T. Farooq, D. N. Barstad, D. L. Blankenhorn, and J. L. Slonczewski**; J. Bacteriol. 178; (1996) 3978-3981; A glutamate-dependent acid resistance gene in *Escherichia coli*

44. **T. Kaneko, S, Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata**; J. DNA Res. 3 (1996) 109-136; Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions

45. **P. Klein, M. Kanehisa, and C. DeLisi**; Biochim. Biophys. Acta 815 (1985) 468-476; The detection and classification of membrane spanning proteins

46. **S. Meng, and G.N. Bennett**; J. Bacteriol. 174 (1992) 2659-2669; Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH

47. **S.R. Waterman, and P.L.C. Small**; Mol. Microbiol. 21 (1996) 925-940; Identification of $\sigma^S$-dependent genes associated with the stationary-phase acid-resistance phenotype of *Shigella flexneri*

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(ii) TITLE OF INVENTION: Salt-inducible promoter derivable from a lactic acid bacterium

(iii) NUMBER OF SEQUENCES: 25

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: .........

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer NS3-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(A) DESCRIPTION: /desc = "synthetic DNA"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer NS3-5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(D) TOPOLOGY: linear

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(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:
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(2) INFORMATION FOR SEQ ID NO: 9:

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(vii) IMMEDIATE SOURCE:
(B) CLONE: primer NS3-11

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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(2) INFORMATION FOR SEQ ID NO: 10:

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:3..500
(D) OTHER INFORMATION:/product= "C-terminus of rnhB3"

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(D) OTHER INFORMATION:/product= "rggL"

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Ser Lys Lys Val Pro Lys Ser Lys His His Ala Ile Leu Ser Glu Ile
                20                  25                  30
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Gln Glu Lys Ala Leu Ala Ile Gly Val Gly Ile Val Asp Ala Glu Lys
            35                  40                  45

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Ile Asp Glu Val Asn Ile Tyr Glu Ala Thr Lys Ile Ala Met Ile Gln
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Ala Val Ser Lys Leu Ser Leu Lys Pro Glu His Leu Leu Ile Asp Ala
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 80                  85                  90                  95

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Phe Glu His Asn Ala Gly Tyr Gly Thr Ala Lys His Leu Ala Ala Leu
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Thr Lys Tyr Gly Ile Thr Arg Ile His Arg Lys Ser Tyr Glu Pro Ile
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Lys Ser Met Val Asn Phe Lys ***
160                 165

GAACGATATT TGGTCACGTT TTTTTGCTGA CAAGTCTGTC AGTAATTATT TTCAAAGGTT   593

TCAAAAATAT AGTCTAGTAA TTTGCTAGAC TAATCTTCTG TTTTTTAATA ATAAACTAAT   653

TTTTTGTTAA TCTAAATGAC AAAATTAATA AGCAGAGTTT TTTATAAAAT TAGCTACTTA   713

TAAAAAATTT GAAATTGGTA TAGTTAAATC TGTTATAATT TCCAATATTT TTTAATAATA   773

ATTATTTTAA CAAAATACTT ATATCAAAAC TCTTTCAAAG TATATAATGA GCGTTGTATA   833

AGCTTTTATG TCTTTCTATA TCAACTTTTA ATAGAAATAT AAAGTAATAT AAATGTTTTT   893

ATAATAAATT ATGTGAGATA TATTTTTTTG TCCGTACTGG TATAGATTTG ACGATTAAGT   953

CTTAAATAAG TTATAATCTC AATTGCGTAA TTTCTTAAAT ACAGAAATAA CAACTACATT  1013

GGTAGACTGA TTAAAAAGTG TACTTGATGA ACTGTTATAA ACCTTAAAAA AATAAAAATA  1073

ATAGTTTGGG GGATGTTAAA G ATG TAT AAA AAA TAT GGA GAT TGT TTT AAA     1124
                        Met Tyr Lys Lys Tyr Gly Asp Cys Phe Lys
                                         5                  10

AAG TTG CGA AAC CAA AAG AAT TTA GGG TTA TCA TAC TTT AGT AAA CTT      1172
Lys Leu Arg Asn Gln Lys Asn Leu Gly Leu Ser Tyr Phe Ser Lys Leu
                15                  20                  25

GGA ATA GAC CGT TCA AAT ATA TCT AGA TTT GAA CAT GGA AAA TGT ATG      1220
Gly Ile Asp Arg Ser Asn Ile Ser Arg Phe Glu His Gly Lys Cys Met
            30                  35                  40

ATG AGT TTT GAG CGT ATA GAT TTG ATG TTA GAA GAA ATG CAA GTT CCG      1268
Met Ser Phe Glu Arg Ile Asp Leu Met Leu Glu Glu Met Gln Val Pro
        45                  50                  55

TTA TCT GAG TAC GAA TTG ATT GTA AAT AAT TTT ATG CCG AAT TTC CAA      1316
Leu Ser Glu Tyr Glu Leu Ile Val Asn Asn Phe Met Pro Asn Phe Gln
    60                  65                  70
```

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GAA TTT TTT ATA TTA GAA TTG GAA AAA GCT GAA TTT AGC CAA AAT CGA     1364
Glu Phe Phe Ile Leu Glu Leu Glu Lys Ala Glu Phe Ser Gln Asn Arg
 75                 80                  85                  90

GAT AAA ATA AAA GAG TTG TAT TCT GAG GTC AAA GAA ACG GGG AAT CAT     1412
Asp Lys Ile Lys Glu Leu Tyr Ser Glu Val Lys Glu Thr Gly Asn His
                95                  100                 105

TTA CTG ACG GTT ACC GTG AAA ACG AAG CTT GGG AAT ATA AGT CAG ACA     1460
Leu Leu Thr Val Thr Val Lys Thr Lys Leu Gly Asn Ile Ser Gln Thr
            110                 115                 120

GAA GTT AAA GAA ATT GAA GCT TAT CTT TGC AAT ATT GAA GAG TGG GGA     1508
Glu Val Lys Glu Ile Glu Ala Tyr Leu Cys Asn Ile Glu Glu Trp Gly
        125                 130                 135

TAT TTT GAA CTT ACT TTA TTT TAT TTT GTA TCT GAT TAT CTC AAT GTC     1556
Tyr Phe Glu Leu Thr Leu Phe Tyr Phe Val Ser Asp Tyr Leu Asn Val
    140                 145                 150

AAT CAA TTA GAA TTG CTG CTT TTT AAT TTT GAT AAA AGA TGT GAA AAT     1604
Asn Gln Leu Glu Leu Leu Leu Phe Asn Phe Asp Lys Arg Cys Glu Asn
155                 160                 165                 170

TAC TGT AGA GTC TTA AAA TAT AGA AGG AGA CTA TTG CAA ATA GCC TAT     1652
Tyr Cys Arg Val Leu Lys Tyr Arg Arg Arg Leu Leu Gln Ile Ala Tyr
                175                 180                 185

AAA AGT GTT GCG ATA TAC GCG GCT AAA GGA GAA AGA AAA AAA GCC GAA     1700
Lys Ser Val Ala Ile Tyr Ala Ala Lys Gly Glu Arg Lys Lys Ala Glu
            190                 195                 200

AAT ATT TTA GAA ATG ACT AAA AAA TAT CGA ACT GTG GGA GTC GAT TTA     1748
Asn Ile Leu Glu Met Thr Lys Lys Tyr Arg Thr Val Gly Val Asp Leu
        205                 210                 215

TAT TCA GAA GTA TTA AGA CAT CTT GCT AGA GCT ATC ATT ATT TTT AAT     1796
Tyr Ser Glu Val Leu Arg His Leu Ala Arg Ala Ile Ile Ile Phe Asn
    220                 225                 230

TTT GAA AAT GCA GAG ATT GGG GAA GAA AAA ATA AAT TAT GCT CTT GAG     1844
Phe Glu Asn Ala Glu Ile Gly Glu Glu Lys Ile Asn Tyr Ala Leu Glu
235                 240                 245                 250

ATT TTG GAA GAA TTT GGA GGA AAG AAG ATA AAA GAA TTC TAT CAG AAT     1892
Ile Leu Glu Glu Phe Gly Gly Lys Lys Ile Lys Glu Phe Tyr Gln Asn
                255                 260                 265

AAA ATG GAA AAG TAT TTG AAA AGG TCA ATT TAG TCTCTTTTGA GCTGTTGCTT 1945
Lys Met Glu Lys Tyr Leu Lys Arg Ser Ile ***
            270                 275

TAAAGCAACA GCTCAAAAGA GATTTTCTTT ATTCTAGAGC ATATACTAGA GGGTGAAGAT 2005

AGGTTGTCTG AAGCATTATA ACTTGTCTTT TAAAAAATTC AATCATAAAT ATAAGGAGGT 2065

ATG ATG AAT CAA AAA AAA TTA TCA TTA TTC GGT TTT TTC GCA TTA ACC     2113
    Met Asn Gln Lys Lys Leu Ser Leu Phe Gly Phe Phe Ala Leu Thr
                    5                  10                  15

GCT TCA ATG GTT TTG ACT GTC TAT GAG TAT CCG ACT TTT GCC ACG TCA     2161
Ala Ser Met Val Leu Thr Val Tyr Glu Tyr Pro Thr Phe Ala Thr Ser
                20                  25                  30

AAA TTA CAT TTG GTG TTC TTT TTA CTT CTC GGA GGA CTA CTA TGG TTT     2209
Lys Leu His Leu Val Phe Phe Leu Leu Leu Gly Gly Leu Leu Trp Phe
            35                  40                  45
```

```
TTG CCT GTA GCG CTC TGC GCA GCA GAA ATG GCG ACG GTT GAA GGC TGG    2257
Leu Pro Val Ala Leu Cys Ala Ala Glu Met Ala Thr Val Glu Gly Trp
         50                  55                  60

AAA AAT GGT GGA ATC TTT AGT TGG GTC AGT CAA ACT TTA GGT GAG CGC    2305
Lys Asn Gly Gly Ile Phe Ser Trp Val Ser Gln Thr Leu Gly Glu Arg
     65                  70                  75

TTT GGT TTT GCA GCC ATA TTT TTT CAG TGG TTC CAA ATT ACA GTA GGT    2353
Phe Gly Phe Ala Ala Ile Phe Phe Gln Trp Phe Gln Ile Thr Val Gly
 80                  85                  90                  95

TTT GTC ACT ATG ATC TAT TTC ATT TTA GGG GCC CTC TCT TAT GTG TTA    2401
Phe Val Thr Met Ile Tyr Phe Ile Leu Gly Ala Leu Ser Tyr Val Leu
                100                 105                 110

AAT TTT CAG GCG CTC AAT ACA GAT CCA TTG ATA AAA TTT ATT GGT TTA    2449
Asn Phe Gln Ala Leu Asn Thr Asp Pro Leu Ile Lys Phe Ile Gly Leu
            115                 120                 125

CTA ATC ATT TTT TGG GGA TTG ACT TTT TCT CAA TTA GGT GGG ACG CAA    2497
Leu Ile Ile Phe Trp Gly Leu Thr Phe Ser Gln Leu Gly Gly Thr Gln
        130                 135                 140

CGG ACT GCC AAA TTA GTA AAA GCT GGC TTT GTA GTT GGA ATA GTG ATT    2545
Arg Thr Ala Lys Leu Val Lys Ala Gly Phe Val Val Gly Ile Val Ile
    145                 150                 155

CCA TCG GTT ATC TTG TTT GGA TTA GCA GCG GCA TAC TTT ATC GGA GGC    2593
Pro Ser Val Ile Leu Phe Gly Leu Ala Ala Ala Tyr Phe Ile Gly Gly
160                 165                 170                 175

AAT CCT ATA GAA ATA CCA ATT AAC AGC CAT GCT TTT GTA CCA GAT TTT    2641
Asn Pro Ile Glu Ile Pro Ile Asn Ser His Ala Phe Val Pro Asp Phe
                180                 185                 190

TCA CAG GTA TCA ACT TTA GTA GTT TTT GTT TCT TTT ATT CTG GCT TAT    2689
Ser Gln Val Ser Thr Leu Val Val Phe Val Ser Phe Ile Leu Ala Tyr
            195                 200                 205

ATG GGG GTA GAA GCC TCA GCT TCA CAT ATT AAT GAA CTT GAA AAT CCA    2737
Met Gly Val Glu Ala Ser Ala Ser His Ile Asn Glu Leu Glu Asn Pro
        210                 215                 220

AAA CGA AAT TAT CCC TTA GCA ATG ATT TTA TTA GTA ATT TTG GCT ATT    2785
Lys Arg Asn Tyr Pro Leu Ala Met Ile Leu Leu Val Ile Leu Ala Ile
    225                 230                 235

TCT TTA GAT GCC ATA GGT GGA TTT TCT GTA GCA GCA GTT ATT CCT CAA    2833
Ser Leu Asp Ala Ile Gly Gly Phe Ser Val Ala Ala Val Ile Pro Gln
240                 245                 250                 255

AAA GAG TTA TCA TTA AGT GCA GGG GTA ATC CAA ACT TTT CAA ACG TTA    2881
Lys Glu Leu Ser Leu Ser Ala Gly Val Ile Gln Thr Phe Gln Thr Leu
                260                 265                 270

ATC TTA CAT TTT AAT CAT CAT TTG GGA TGG TTA GTT AAA GTG ATT GCA    2929
Ile Leu His Phe Asn His His Leu Gly Trp Leu Val Lys Val Ile Ala
            275                 280                 285

CTA ATG ATT GCC TTT GGG GTT ATG GGA GAA GTG AGT TCA TGG GTT GTT    2977
Leu Met Ile Ala Phe Gly Val Met Gly Glu Val Ser Ser Trp Val Val
        290                 295                 300

GGT CCT TCT AGA 2989
Gly Pro Ser Arg
    305
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 276 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein rggL ex *L. lactis* MG1363

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
MYKKYGDCFK KLRNQKNLGL SYFSKLGIDR SNISRFEHGK CMMSFERIDL    50
MLEEMQVPLS EYELIVNNFM PNFQEFFILE LEKAEFSQNR DKIKELYSEV   100
KETGNHLLTV TVKTKLGNIS QTEVKEIEAY LCNIEEWGYF ELTLFYFVSD   150
YLNVNQLELL LFNFDKRCEN YCRVLKYRRR LLQIAYKSVA IYAAKGERKK   200
AENILEMTKK YRTVGVDLYS EVLRHLARAI IIFNFENAEI GEEKINYALE   250
ILEEFGGKKI KEFYQNKMEK YLKRSI                             276
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 297 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein rgg1 ex *S. gordonii*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
MLIVKSSGKI LKIIRESKNM SLKEVAAGDI SVAQLSRYER GISSLTVDSF    50
YSCLRNMSVS LAEFQYVYHN YREADDVVLS QKLSEAQREN NIVKLESILA   100
GSEAMAQEFP EKKNYKLNTI VIRATLTSCN PDYQVSKGDI EFLTDYLFSV   150
EEWGRYELWL FTNSVNLLTL ETLETFASEM INRTQFYNNL PENRRRIIKM   200
LLNVVSACIE NNHLQVAMKF LNYIDNTKIP ETDLYDRVLI KYHKALYSYK   250
VGNPHARHDI EQCLSTFEYL DSFGVARKLK EQFERIQLTV VADLQIE      297
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein ORF3 ex *L. lactis* C2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
MPYKRYGEIF KKLREQKNFS LSHFSEIGIS KASLSRFELG QTMISFERLD    50
SALQEMNVTL AEYEHFINNF SMDYKEEFLE DIILADIADD VDKLHKL       97
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 166 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein C-terminus of rnhB ex *L. lactis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
AVILPKNCKI RGLNDSKKVP KSKHHAILSE IQEKALAIGV GIVDAEKIDE    50
VNIYEATKIA MIQAVSKLSL KPEHLLIDAM VLDLPIAQTK IIHGDARSAS   100
IAAASIVAKV TRDEMMKDFA LEFPEYDFEH NAGYGTAKHL AALTKYGITR   150
IHRKSYEPIK SMVNFK                                       166
```

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 161 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein C-terminus of rnhB ex *Vibrio cholera* (U30472)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
PNRPIMGLND SKKLSEKKRL ALFPEIQVKA LAWAVGRCSP QEIDELNIFQ    50
ATMVAMQRAV AGLRIQPDLV LIDGNKIPKL PMEAQAVVKG DLRVAQISAA   100
SIIAKVIRDQ EMEALDKQYP QFGFANHKGY PTAAHFAAIE QHGVIEQHRK   150
SFGPVKRALG E                                             161
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 213 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein rnhB ex *E. coli*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
MIEFVYPHTQ LVAGVDEVGR GPLVGAVVTA AVILDPARPI AGLNDSKKLS    50
EKRRLALYEE IKEKALSWSL GRAEPHEIDE LNILHATMLA MQRAVAGLHI   100
APEYVLIDGN RCPKLPMPAM AVVKGDSRVP EISAASILAK VTRDAEMAAL   150
DIVFPQYGFA QHKGYPTAFH LEKLAEHGAT EHHRRSFGPV KRALGTCVLI   200
LVSRLSKPES EDV                                           213
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 197 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein rnhB ex *Haemophilus influenza*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
MFEYPQGYKL IAGVDEVGRG PLVGAVVTAA VILDPHNPIE GLADSKKLSE    50
KKRLALAEEI KEKARAWALG RAEADEIDEI NILQASLLAM TRAVKSLKIQ   100
PHFVLIDGNK IPKDLAIPAQ AVVKGDSLVA EISAASILAK VARDQEMEEL   150
DKQYPEYAFA QHKGYPTKLH LEKLAELGAL PQHRRSFAPV KKALEQF      197
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "fusion point of truncated *orfX* with *lytPR* in pNS3PR"

(vii) IMMEDIATE SOURCE:
(B) CLONE: pNS3PR

(ix) FEATURE:
(A) NAME/KEY:mat_peptide
(B) LOCATION:92..283

(D) OTHER INFORMATION:/product= "C-terminus of ORFX"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:297..359
(D) OTHER INFORMATION:/product= "lytP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
TCTAGAGCAT ATACTAGAGG GTGAAGATAG GTTGTCTGAA GCATTATAAC TTGTCTTTTA      60

AAAAATTCAA TCATAAATAT AAGGAGGTAT G ATG AAG TAC TGG ATA CTT ATC        112
                                   Met Lys Tyr Trp Ile Leu Ile
                                                       5

AAT GAT GAA CCT TGG TTT GTC GGA AAA GAT GTA GCA ATT GCT ATT GGT       160
Asn Asp Glu Pro Trp Phe Val Gly Lys Asp Val Ala Ile Ala Ile Glu
        10                  15                  20

TAC AAG AAT TTC AGG GAT GCT TTG AAA TCT CAT GTA AAA GAC AAA TAT       208
Tyr Lys Asn Phe Arg Asp Ala Leu Lys Ser His Val Lys Asp Lys Tyr
    25                  30                  35

AAG AGG GAG TCG GAC AGC AGT GAT TGG TTC AAC GAC AAT ATA TTA TTG       256
Lys Arg Glu Ser Asp Ser Ser Asp Trp Phe Asn Asp Asn Ile Leu Leu
40                  45                  50                  55

GAA ACG AAC TGC ATA AAA AAT AAA AAA TAG GAGAAAGAAC ATG AAA ACA        305
Glu Thr Asn Cys Ile Lys Asn Lys Lys ***            Met Lys Thr
                60

TTT TTT AAA GAT ATG GCA GAA CGT GCC ATT AAA ACA TTT GCA CAA GCA       353
Phe Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe Ala Gln Ala
    5                   10                  15

ATG ATT G                                                             360
Met Ile
20
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "fusion point of truncated *orfX* with *acmA* in pNS3AL3"

(vii) IMMEDIATE SOURCE:
(B) CLONE: pNS3AL3

(ix) FEATURE:
(A) NAME/KEY:mat_peptide
(B) LOCATION:92..154
(D) OTHER INFORMATION:/product= "truncated ORFX"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
TCTAGAGCAT ATACTAGAGG GTGAAGATAG GTTGTCTGAA GCATTATAAC TTGTCTTTTA      60

AAAGATTCAA TCATAAATAT AAGGAGGTAT G ATG AAG TAC TTA TTA TAT TTT        112
                                   Met Lys Tyr Leu Leu Tyr Phe
                                                       5
```

```
GTA ATC TTT AGA AAG GTA ATT ATT TAT GCC AGT ATC ACG TGT TAA          157
Val Ile Phe Arg Lys Val Ile Ile Tyr Ala Ser Ile Thr Cys ***
         10                  15                  20

AGTTAAAAAT AGACATTTAA AAA                                            180
```

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 179 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
- (A) DESCRIPTION: /desc = "fusion point of truncated *orfX* with *acmA* in pNS3AL3"

(vii) IMMEDIATE SOURCE:
- (B) CLONE: pNS3AL3

(ix) FEATURE:
- (A) NAME/KEY:mat_peptide
- (B) LOCATION:92..112
- (D) OTHER INFORMATION:/product= "truncated ORFX"

(ix) FEATURE:
- (A) NAME/KEY:mat_peptide
- (B) LOCATION:137..178
- (D) OTHER INFORMATION:/product= "N-terminus of acmA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```
TCTAGAGCAT ATACTAGAGG GTGAAGATAG GTTGTCTGAA GCATTATAAC TTGTCTTTTA     60

AAAGATTCAA TCATAAATAT AAGGAGGTAT G ATG AAG TCT TAT TAT ATT TTG TAA   115
                                   Met Lys Ser Tyr Tyr Ile Leu ***
                                                     5
TCTTTAGAAA GGTAATTATT T ATG CCA GTA TCA CGT GTT AAA GTT AAA AAT AGA  169
                        Met Pro Val Ser Arg Val Lys Val Lys Asn Arg
                                          5                   10
CAT TTA AAA A                                                        179
His Leu Lys
```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 5565 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
- (A) DESCRIPTION: /desc = "genomic DNA of NS3 locus containing *gadRCB*"

(vii) IMMEDIATE SOURCE:
- (B) CLONE: *Pst*I-*Eco*RI fragment

(ix) FEATURE:
- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 3..500
- (D) OTHER INFORMATION: /product= "C-terminus of rnhB"

(ix) FEATURE:
- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 1095..1922
- (D) OTHER INFORMATION: /product= "rggL = gadR"

```
(ix) FEATURE:
      (A) NAME/KEY: mat_peptide
      (B) LOCATION: 2069..3577
      (D) OTHER INFORMATION:/product= "orfX = gadC"

(ix) FEATURE:
      (A) NAME/KEY: mat_peptide
      (B) LOCATION: 3600..4997
      (D) OTHER INFORMATION:/product= "gadB"

(ix) FEATURE:
      (A) NAME/KEY: mat_peptide
      (B) LOCATION: 5563..5078
      (D) OTHER INFORMATION:/product= "C-terminus of unknown ORF in
                                       opposite direction"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CT GCA GTA ATT TTG CCA AAG AAT TGT AAA ATT CGT GGT TTG AAT GAT      47
   Ala Val Ile Leu Pro Lys Asn Cys Lys Ile Arg Gly Leu Asn Asp
                   5                  10                  15

AGT AAA AAA GTG CCA AAA TCA AAG CAT CAT GCT ATT CTA TCT GAA ATT     95
Ser Lys Lys Val Pro Lys Ser Lys His His Ala Ile Leu Ser Glu Ile
                20                  25                  30
CAA GAA AAA GCG CTA GCG ATT GGA GTT GGA ATT GTT GAT GCC GAA AAA    143
Gln Glu Lys Ala Leu Ala Ile Gly Val Gly Ile Val Asp Ala Glu Lys
            35                  40                  45

ATT GAT GAA GTA AAT ATT TAT GAG GCG ACA AAA ATT GCA ATG ATT CAG    191
Ile Asp Glu Val Asn Ile Tyr Glu Ala Thr Lys Ile Ala Met Ile Gln
        50                  55                  60

GCA GTA TCA AAA TTA TCT CTT AAA CCT GAA CAT CTC TTA ATA GAT GCG    239
Ala Val Ser Lys Leu Ser Leu Lys Pro Glu His Leu Leu Ile Asp Ala
    65                  70                  75

ATG GTT TTA GAT TTG CCC ATT GCT CAG ACG AAA ATC ATT CAT GGA GAT    287
Met Val Leu Asp Leu Pro Ile Ala Gln Thr Lys Ile Ile His Gly Asp
80                  85                  90                  95

GCT CGT TCA GCT TCA ATT GCG GCC GCA TCA ATT GTA GCT AAA GTG ACT    335
Ala Arg Ser Ala Ser Ile Ala Ala Ala Ser Ile Val Ala Lys Val Thr
                100                 105                 110

CGT GAT GAA ATG ATG AAG GAT TTC GCT TTA GAA TTT CCA GAA TAT GAT    383
Arg Asp Glu Met Met Lys Asp Phe Ala Leu Glu Phe Pro Glu Tyr Asp
            115                 120                 125

TTT GAA CAT AAT GCA GGC TAT GGA ACA GCA AAA CAT CTT GCA GCT CTG    431
Phe Glu His Asn Ala Gly Tyr Gly Thr Ala Lys His Leu Ala Ala Leu
        130                 135                 140

ACA AAA TAT GGT ATC ACA AGA ATT CAT CGG AAA TCC TAT GAA CCA ATT    479
Thr Lys Tyr Gly Ile Thr Arg Ile His Arg Lys Ser Tyr Glu Pro Ile
    145                 150                 155

AAA TCG ATG GTC AAT TTC AAA TAG TAGATTATGT AAGTAAAAAA AGGAAAACGT   533
Lys Ser Met Val Asn Phe Lys ***
160                 165

GAACGATATT TGGTCACGTT TTTTTGCTGA CAAGTCTGTC AGTAATTATT TTCAAAGGTT  593

TCAAAAATAT AGTCTAGTAA TTTGCTAGAC TAATCTTCTG TTTTTTAATA ATAAACTAAT  653

TTTTTGTTAA TCTAAATGAC AAAATTAATA AGCAGAGTTT TTTATAAAAT TAGCTACTTA  713

TAAAAAATTT GAAATTGGTA TAGTTAAATC TGTTATAATT TCCAATATTT TTTAATAATA  773
```

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ATTATTTTAA CAAAATACTT ATATCAAAAC TCTTTCAAAG TATATAATGA GCGTTGTATA  833

AGCTTTTATG TCTTTCTATA TCAACTTTTA ATAGAAATAT AAAGTAATAT AAATGTTTTT  893

ATAATAAATT ATGTGAGATA TATTTTTTTG TCCGTACTGG TATAGATTTG ACGATTAAGT  953

CTTAAATAAG TTATAATCTC AATTGCGTAA TTTCTTAAAT ACAGAAATAA CAACTACATT 1013

GGTAGACTGA TTAAAAAGTG TACTTGATGA ACTGTTATAA ACCTTAAAAA AATAAAAATA 1073

ATAGTTTGGG GGATGTTAAA G ATG TAT AAA AAA TAT GGA GAT TGT TTT AAA   1124
                        Met Tyr Lys Lys Tyr Gly Asp Cys Phe Lys
                                          5                  10

AAG TTG CGA AAC CAA AAG AAT TTA GGG TTA TCA TAC TTT AGT AAA CTT   1172
Lys Leu Arg Asn Gln Lys Asn Leu Gly Leu Ser Tyr Phe Ser Lys Leu
             15                  20                  25

GGA ATA GAC CGT TCA AAT ATA TCT AGA TTT GAA CAT GGA AAA TGT ATG   1220
Gly Ile Asp Arg Ser Asn Ile Ser Arg Phe Glu His Gly Lys Cys Met
         30                  35                  40

ATG AGT TTT GAG CGT ATA GAT TTG ATG TTA GAA GAA ATG CAA GTT CCG   1268
Met Ser Phe Glu Arg Ile Asp Leu Met Leu Glu Glu Met Gln Val Pro
     45                  50                  55

TTA TCT GAG TAC GAA TTG ATT GTA AAT AAT TTT ATG CCG AAT TTC CAA   1316
Leu Ser Glu Tyr Glu Leu Ile Val Asn Asn Phe Met Pro Asn Phe Gln
 60                  65                  70

GAA TTT TTT ATA TTA GAA TTG GAA AAA GCT GAA TTT AGC CAA AAT CGA   1364
Glu Phe Phe Ile Leu Glu Leu Glu Lys Ala Glu Phe Ser Gln Asn Arg
75                  80                  85                  90

GAT AAA ATA AAA GAG TTG TAT TCT GAG GTC AAA GAA ACG GGG AAT CAT   1412
Asp Lys Ile Lys Glu Leu Tyr Ser Glu Val Lys Glu Thr Gly Asn His
             95                 100                 105

TTA CTG ACG GTT ACC GTG AAA ACG AAG CTT GGG AAT ATA AGT CAG ACA   1460
Leu Leu Thr Val Thr Val Lys Thr Lys Leu Gly Asn Ile Ser Gln Thr
        110                 115                 120

GAA GTT AAA GAA ATT GAA GCT TAT CTT TGC AAT ATT GAA GAG TGG GGA   1508
Glu Val Lys Glu Ile Glu Ala Tyr Leu Cys Asn Ile Glu Glu Trp Gly
    125                 130                 135

TAT TTT GAA CTT ACT TTA TTT TAT TTT GTA TCT GAT TAT CTC AAT GTC   1556
Tyr Phe Glu Leu Thr Leu Phe Tyr Phe Val Ser Asp Tyr Leu Asn Val
140                 145                 150

AAT CAA TTA GAA TTG CTG CTT TTT AAT TTT GAT AAA AGA TGT GAA AAT   1604
Asn Gln Leu Glu Leu Leu Leu Phe Asn Phe Asp Lys Arg Cys Glu Asn
155                 160                 165                 170

TAC TGT AGA GTC TTA AAA TAT AGA AGG AGA CTA TTG CAA ATA GCC TAT   1652
Tyr Cys Arg Val Leu Lys Tyr Arg Arg Arg Leu Leu Gln Ile Ala Tyr
            175                 180                 185

AAA AGT GTT GCG ATA TAC GCG GCT AAA GGA GAA AGA AAA AAA GCC GAA   1700
Lys Ser Val Ala Ile Tyr Ala Ala Lys Gly Glu Arg Lys Lys Ala Glu
        190                 195                 200

AAT ATT TTA GAA ATG ACT AAA AAA TAT CGA ACT GTG GGA GTC GAT TTA   1748
Asn Ile Leu Glu Met Thr Lys Lys Tyr Arg Thr Val Gly Val Asp Leu
    205                 210                 215
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TAT TCA GAA GTA TTA AGA CAT CTT GCT AGA GCT ATC ATT ATT TTT AAT    1796
Tyr Ser Glu Val Leu Arg His Leu Ala Arg Ala Ile Ile Ile Phe Asn
    220                 225                 230

TTT GAA AAT GCA GAG ATT GGG GAA GAA AAA ATA AAT TAT GCT CTT GAG    1844
Phe Glu Asn Ala Glu Ile Gly Glu Glu Lys Ile Asn Tyr Ala Leu Glu
235                 240                 245                 250

ATT TTG GAA GAA TTT GGA GGA AAG AAG ATA AAA GAA TTC TAT CAG AAT    1892
Ile Leu Glu Glu Phe Gly Gly Lys Lys Ile Lys Glu Phe Tyr Gln Asn
                255                 260                 265

AAA ATG GAA AAG TAT TTG AAA AGG TCA ATT TAG TCTCTTTTGA GCTGTTGCTT 1945
Lys Met Glu Lys Tyr Leu Lys Arg Ser Ile ***
            270                 275

TAAAGCAACA GCTCAAAAGA GATTTTCTTT ATTCTAGAGC ATATACTAGA GGGTGAAGAT 2005

AGGTTGTCTG AAGCATTATA ACTTGTCTTT TAAAAAATTC AATCATAAAT ATAAGGAGGT 2065

ATG ATG AAT CAA AAA AAA TTA TCA TTA TTC GGT TTT TTC GCA TTA ACC    2113
    Met Asn Gln Lys Lys Leu Ser Leu Phe Gly Phe Phe Ala Leu Thr
                    5                  10                  15

GCT TCA ATG GTT TTG ACT GTC TAT GAG TAT CCG ACT TTT GCC ACG TCA    2161
Ala Ser Met Val Leu Thr Val Tyr Glu Tyr Pro Thr Phe Ala Thr Ser
                20                  25                  30

AAA TTA CAT TTG GTG TTC TTT TTA CTT CTC GGA GGA CTA CTA TGG TTT    2209
Lys Leu His Leu Val Phe Phe Leu Leu Leu Gly Gly Leu Leu Trp Phe
            35                  40                  45

TTG CCT GTA GCG CTC TGC GCA GCA GAA ATG GCG ACG GTT GAA GGC TGG    2257
Leu Pro Val Ala Leu Cys Ala Ala Glu Met Ala Thr Val Glu Gly Trp
        50                  55                  60

AAA AAT GGT GGA ATC TTT AGT TGG GTC AGT CAA ACT TTA GGT GAG CGC    2305
Lys Asn Gly Gly Ile Phe Ser Trp Val Ser Gln Thr Leu Gly Glu Arg
    65                  70                  75

TTT GGT TTT GCA GCC ATA TTT TTT CAG TGG TTC CAA ATT ACA GTA GGT    2353
Phe Gly Phe Ala Ala Ile Phe Phe Gln Trp Phe Gln Ile Thr Val Gly
80                  85                  90                  95

TTT GTC ACT ATG ATC TAT TTC ATT TTA GGG GCC CTC TCT TAT GTG TTA    2401
Phe Val Thr Met Ile Tyr Phe Ile Leu Gly Ala Leu Ser Tyr Val Leu
                100                 105                 110

AAT TTT CAG GCG CTC AAT ACA GAT CCA TTG ATA AAA TTT ATT GGT TTA    2449
Asn Phe Gln Ala Leu Asn Thr Asp Pro Leu Ile Lys Phe Ile Gly Leu
            115                 120                 125

CTA ATC ATT TTT TGG GGA TTG ACT TTT TCT CAA TTA GGT GGG ACG CAA    2497
Leu Ile Ile Phe Trp Gly Leu Thr Phe Ser Gln Leu Gly Gly Thr Gln
        130                 135                 140

CGG ACT GCC AAA TTA GTA AAA GCT GGC TTT GTA GTT GGA ATA GTG ATT    2545
Arg Thr Ala Lys Leu Val Lys Ala Gly Phe Val Val Gly Ile Val Ile
    145                 150                 155

CCA TCG GTT ATC TTG TTT GGA TTA GCA GCG GCA TAC TTT ATC GGA GGC    2593
Pro Ser Val Ile Leu Phe Gly Leu Ala Ala Ala Tyr Phe Ile Gly Gly
160                 165                 170                 175

AAT CCT ATA GAA ATA CCA ATT AAC AGC CAT GCT TTT GTA CCA GAT TTT    2641
Asn Pro Ile Glu Ile Pro Ile Asn Ser His Ala Phe Val Pro Asp Phe
                180                 185                 190
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TCA CAG GTA TCA ACT TTA GTA GTT TTT GTT TCT TTT ATT CTG GCT TAT 2689
Ser Gln Val Ser Thr Leu Val Val Phe Val Ser Phe Ile Leu Ala Tyr
195 200 205

ATG GGG GTA GAA GCC TCA GCT TCA CAT ATT AAT GAA CTT GAA AAT CCA 2737
Met Gly Val Glu Ala Ser Ala Ser His Ile Asn Glu Leu Glu Asn Pro
210 215 220

AAA CGA AAT TAT CCC TTA GCA ATG ATT TTA TTA GTA ATT TTG GCT ATT 2785
Lys Arg Asn Tyr Pro Leu Ala Met Ile Leu Leu Val Ile Leu Ala Ile
225 230 235

TCT TTA GAT GCC ATA GGT GGA TTT TCT GTA GCA GCA GTT ATT CCT CAA 2833
Ser Leu Asp Ala Ile Gly Gly Phe Ser Val Ala Ala Val Ile Pro Gln
240 245 250 255

AAA GAG TTA TCA TTA AGT GCA GGG GTA ATC CAA ACT TTT CAA ACG TTA 2881
Lys Glu Leu Ser Leu Ser Ala Gly Val Ile Gln Thr Phe Gln Thr Leu
260 265 270

ATC TTA CAT TTT AAT CAT CAT TTG GGA TGG TTA GTT AAA GTG ATT GCA 2929
Ile Leu His Phe Asn His His Leu Gly Trp Leu Val Lys Val Ile Ala
275 280 285

CTA ATG ATT GCC TTT GGG GTT ATG GGA GAA GTG AGT TCA TGG GTT GTT 2977
Leu Met Ile Ala Phe Gly Val Met Gly Glu Val Ser Ser Trp Val Val
290 295 300

GGT CCT TCT AGA GGG ATG TTT GCA GCA GCA CAA AGA GGT TTA TTA CCA 3025
Gly Pro Ser Arg Gly Met Phe Ala Ala Ala Gln Arg Gly Leu Leu Pro
305 310 315

AAA TTT TTA CGT AAA ACG AAT ACA CAT GAA GTC CCT GTT CCT TTA GTT 3073
Lys Phe Leu Arg Lys Thr Asn Thr His Glu Val Pro Val Pro Leu Val
320 325 330 335

ATG ATT CAA GGA ATC ATT GTT ACA CTT TGG GGC GCT GTA TTA ACT TTT 3121
Met Ile Gln Gly Ile Ile Val Thr Leu Trp Gly Ala Val Leu Thr Phe
340 345 350

GGA GGA GGA GGA AAT AAT TTA TCT TTC TTA GTT GCC ATT TCA CTG ACT 3169
Gly Gly Gly Gly Asn Asn Leu Ser Phe Leu Val Ala Ile Ser Leu Thr
355 360 365

GTA GTG ATT TAT TTG GTG GGT TAC CTC TTG TTC TTT ATT GTT TAC TTT 3217
Val Val Ile Tyr Leu Val Gly Tyr Leu Leu Phe Phe Ile Val Tyr Phe
370 375 380

GTT TTA ATC TAT AAA AAA CAA AAT TTA AAG CGT ACT TAT AAT GTT CCA 3265
Val Leu Ile Tyr Lys Lys Gln Asn Leu Lys Arg Thr Tyr Asn Val Pro
385 390 395

GGT AAA ATA ATA GGA AAA ACA ATC ATT GCA GGA ATT GGA TTC TTA TTA 3313
Gly Lys Ile Ile Gly Lys Thr Ile Ile Ala Gly Ile Gly Phe Leu Leu
400 405 410 415

TCA ATT TTT GCT CTA TTT ATT TCC TTT GTT CCT CCA GCA TCA ATT GCG 3361
Ser Ile Phe Ala Leu Phe Ile Ser Phe Val Pro Pro Ala Ser Ile Ala
420 425 430

AAA AAT GAA ACT CAC ACC TAT CAA ATG ATA CTT CTT ATA AGT TTT GTT 3409
Lys Asn Glu Thr His Thr Tyr Gln Met Ile Leu Leu Ile Ser Phe Val
435 440 445

GTG ACC GCT ATC TTG CCA TTT ATT ATT TAT GAA TTG CAT GAT AAA AAG 3457
Val Thr Ala Ile Leu Pro Phe Ile Ile Tyr Glu Leu His Asp Lys Lys
450 455 460

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GGA CAT GAT ACT ATT GAA GAA CCA ACA CAC TTT AAA GCA GGA GAT GTG     3505
Gly His Asp Thr Ile Glu Glu Pro Thr His Phe Lys Ala Gly Asp Val
    465                 470                 475

AAC CCT GCG ATT TAT CCA GCA GCT CGT GGA GAG CAT CAT ATT ATT AAA     3553
Asn Pro Ala Ile Tyr Pro Ala Ala Arg Gly Glu His His Ile Ile Lys
480                 485                 490                 495

AAA GAA GAA CAT ATC TTA AAA CAT TGA AAAATTggag gATGTACAT ATG        3602
Lys Glu Glu His Ile Leu Lys His ***                      Met
                500         503

TTA TAC GGA AAA GAA AAT CGA GAT GAA GCG GAG TTC TTG GAA CCA ATT     3650
Leu Tyr Gly Lys Glu Asn Arg Asp Glu Ala Glu Phe Leu Glu Pro Ile
            5                   10                  15

TTT GGT TCA GAA AGT GAA CAA GTG GAT TTA CCT AAA TAT AAA TTA GCT     3698
Phe Gly Ser Glu Ser Glu Gln Val Asp Leu Pro Lys Tyr Lys Leu Ala
        20                  25                  30

CAA CAA TCA ATT GAG CCT CGA GTG GCC TAT CAG TTA GTT CAA GAT GAA     3746
Gln Gln Ser Ile Glu Pro Arg Val Ala Tyr Gln Leu Val Gln Asp Glu
    35                  40                  45

ATG CTA GAT GAA GGG AAC GCT CGT TTA AAT TTG GCC ACA TTC TGT CAA     3794
Met Leu Asp Glu Gly Asn Ala Arg Leu Asn Leu Ala Thr Phe Cys Gln
50                  55                  60                  65

ACT TAT ATG GAA CCT GAA GCA GTC AAG CTG ATG AGT CAG ACC TTG GAA     3842
Thr Tyr Met Glu Pro Glu Ala Val Lys Leu Met Ser Gln Thr Leu Glu
                70                  75                  80

AAA AAT GCG ATT GAC AAA TCA GAA TAT CCA AGA ACA ACT GAA ATT GAA     3890
Lys Asn Ala Ile Asp Lys Ser Glu Tyr Pro Arg Thr Thr Glu Ile Glu
            85                  90                  95

AAC CGT TGC GTC AAC ATG ATC GCT GAC CTT TGG AAT GCG AGT GAA AAA     3938
Asn Arg Cys Val Asn Met Ile Ala Asp Leu Trp Asn Ala Ser Glu Lys
        100                 105                 110

GGA AAA ATT TAT GGG ACT TCG ACA ATT GGT TCT TCA GAA GCT TGT ATG     3986
Gly Lys Ile Tyr Gly Thr Ser Thr Ile Gly Ser Ser Glu Ala Cys Met
    115                 120                 125

CTT GGG GGA ATG GCT ATG AAG TTT TCT TGG CGT AAG CGA GCA GAA AAA     4034
Leu Gly Gly Met Ala Met Lys Phe Ser Trp Arg Lys Arg Ala Glu Lys
130                 135                 140                 145

TTA GGC CTA GAT ATT AAT GCG AAA AAG CCA AAC TTA GTC ATT TCC TCT     4082
Leu Gly Leu Asp Ile Asn Ala Lys Lys Pro Asn Leu Val Ile Ser Ser
                150                 155                 160

GGT TAT CAA GTT TGC TGG GAA AAA TTC TGT GTT TAT TGG GAT ATT GAA     4130
Gly Tyr Gln Val Cys Trp Glu Lys Phe Cys Val Tyr Trp Asp Ile Glu
            165                 170                 175

ATG AGA GAA GTG CCA ATG GAT AGA GAA CAT ATG TCA ATC AAT TTG GAA     4178
Met Arg Glu Val Pro Met Asp Arg Glu His Met Ser Ile Asn Leu Glu
        180                 185                 190

AAA GTG ATG GAT TAT GTT GAT GAA TAT ACG ATT GGA GTA GTT GGA ATT     4226
Lys Val Met Asp Tyr Val Asp Glu Tyr Thr Ile Gly Val Val Gly Ile
    195                 200                 205

ATG GGG ATT ACT TAT ACT GGT CGT TAT GAT GAT ATC AAA GCT TTG GAT     4274
Met Gly Ile Thr Tyr Thr Gly Arg Tyr Asp Asp Ile Lys Ala Leu Asp
210                 215                 220                 225
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AAT TTG ATT GAA GAA TAT AAT AAA CAG ACA GAC TAC AAA GTT TAT ATT     4322
Asn Leu Ile Glu Glu Tyr Asn Lys Gln Thr Asp Tyr Lys Val Tyr Ile      241
                230                 235                 240

CAC GTA GAT GCT GCT TCA GGA GGA CTT TAT GCT CCT TTT GTT GAG CCA     4370
His Val Asp Ala Ala Ser Gly Gly Leu Tyr Ala Pro Phe Val Glu Pro
            245                 250                 255

GAA CTT GAG TGG GAT TTC CGT TTG AAA AAT GTC ATT TCA ATC AAT ACT     4418
Glu Leu Glu Trp Asp Phe Arg Leu Lys Asn Val Ile Ser Ile Asn Thr
        260                 265                 270

TCA GGA CAT AAA TAT GGT TTA GTA TAT CCT GGT GTA GGT TGG GTC TTG     4466
Ser Gly His Lys Tyr Gly Leu Val Tyr Pro Gly Val Gly Trp Val Leu
    275                 280                 285

TGG CGT GAC AAA AAA TAT TTA CCT GAA GAG TTA ATT TTT AAA GTA AGT     4514
Trp Arg Asp Lys Lys Tyr Leu Pro Glu Glu Leu Ile Phe Lys Val Ser
290                 295                 300                 305

TAT CTT GGA GGA GAA TTA CCA ACA ATG GCG ATT AAT TTT TCT CAC AGT     4562
Tyr Leu Gly Gly Glu Leu Pro Thr Met Ala Ile Asn Phe Ser His Ser
                310                 315                 320

GCT TCT CAA TTA ATC GGT CAA TAC TAT AAT TTT GTA CGT TAT GGA TTT     4610
Ala Ser Gln Leu Ile Gly Gln Tyr Tyr Asn Phe Val Arg Tyr Gly Phe
            325                 330                 335

GAT GGA TAT AAA GCT ATT CAT GAG AGA ACG CAT AAA GTA GCC ATG TAT     4658
Asp Gly Tyr Lys Ala Ile His Glu Arg Thr His Lys Val Ala Met Tyr
        340                 345                 350

TTA GCA GAA GAA ATT GAA AAA ACA GGA ATG TTT GAG ATT ATG AAC GAT     4706
Leu Ala Glu Glu Ile Glu Lys Thr Gly Met Phe Glu Ile Met Asn Asp
    355                 360                 365

GGG GCA CAA TTA CCA ATT GTC TGC TAC AAA TTA AAA GAA AAT TCA AAC     4754
Gly Ala Gln Leu Pro Ile Val Cys Tyr Lys Leu Lys Glu Asn Ser Asn
370                 375                 380                 385

CGT GGT TGG AAT CTT TAT GAT TTG GCA GAT CGT TTA TTA ATG AAG GGA     4802
Arg Gly Trp Asn Leu Tyr Asp Leu Ala Asp Arg Leu Leu Met Lys Gly
                390                 395                 400

TGG CAA GTG CCT GCT TAT CCA CTT CCT AAA AAT TTG GAA AAT GAA ATC     4850
Trp Gln Val Pro Ala Tyr Pro Leu Pro Lys Asn Leu Glu Asn Glu Ile
            405                 410                 415

ATT CAA CGT TTA GTA ATT CGA GCA GAT TTC GGG ATG AAT ATG GCA TTT     4898
Ile Gln Arg Leu Val Ile Arg Ala Asp Phe Gly Met Asn Met Ala Phe
        420                 425                 430

AAC TAT GTT CAA GAT ATG CAA GAA GCA ATT GAT GCA CTA AAT AAG GCT     4946
Asn Tyr Val Gln Asp Met Gln Glu Ala Ile Asp Ala Leu Asn Lys Ala
    435                 440                 445

CAT ATT CTA TTT CAT CAG GAA CCT GAA AAT AAA ACA TAT GGC TTT ACT     4994
His Ile Leu Phe His Gln Glu Pro Glu Asn Lys Thr Tyr Gly Phe Thr
450                 455                 460                 465

CAC TAA AGATAAAAGC GATATATCTA AGATATATCG CTTTTATTTT GTTTTAGGCT      5050
His

ATTTACTAAT TAGCTTGTCG CTTA TTA TTT TTC ATA GTA TTT ATC CAA AAT      5101
                          *** Lys Glu Tyr Tyr Lys Asp Leu Ile
                                              -5
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TTC CAT TTT TAA AGG AGT AAT TTT AGA TAG GGG GGC AGT TAG ACT TGT      5149
Glu Met Lys Leu Pro Thr Ile Lys Ser Leu Pro Ala Thr Leu Ser Thr
    -10                 -15                 -20

TCT TAG GAA GAG CTT ATC TTC AAT GTT GAT GAT ACC CAG ATA TTT AAC      5197
Arg Leu Phe Leu Lys Asp Glu Ile Asn Ile Ile Gly Leu Tyr Lys Val
-25                 -30                 -35                 -40

TTG AGG GTA GTT AGC ATT GAC TTC TAT AAT TTG GGC TTT TTT CTC ACT      5245
Gln Pro Tyr Asn Ala Asn Val Glu Ile Ile Gln Ala Lys Lys Glu Ser
                -45                 -50                 -55

AAT ATT TTC GTC TGT CAC GGG CAC ATC TAG GTT GAC CGT TCT TTC TTT      5293
Ile Asn Glu Asp Thr Val Pro Val Asp Leu Asn Val Thr Arg Glu Lys
            -60                 -65                 -70

ATA AGA GTA ATT TTT GAG AGC AGC AAT ATT TCG GTT TGG AAT AAA AGT      5341
Tyr Ser Tyr Asn Lys Leu Ala Ala Ile Asn Arg Asn Pro Ile Phe Thr
        -75                 -80                 -85

AGT CGC ACC GTC GGC TCC GAT AAC AGT AAT GGA ACG AAT TCC TAC GGT      5389
Thr Ala Gly Asp Ala Gly Ile Val Thr Ile Ser Arg Ile Gly Val Thr
    -90                 -95                 -100

TTT CAC CAC TCC CTC AAT ATC AAG ACC GGC AAA GGC AAC CGT ATC CGC      5437
Lys Val Val Gly Glu Ile Asp Leu Gly Ala Phe Ala Val Thr Asp Ala
-105                -110                -115                -120

AAC ATT GAT TTG ATG TTC AAC GAT AAT AAA GAA ACC ATT AAT GAT ATC      5485
Val Asn Ile Gln His Glu Val Ile Ile Phe Phe Gly Asn Ile Ile Asp
                -125                -130                -135

AGC GAC TAA ATC TCG TCC AGC GAA ACC AAG AGC GAC TCC AAG GAT ACC      5533
Ala Val Leu Asp Arg Gly Ala Phe Gly Leu Ala Val Gly Leu Ile Gly
            -140                -145                -150

CGC GCC AGC TAG GAC ATT TGC AAC AGG AATTC                            5565
Ala Gly Ala Leu Val Asn Ala Val Pro Leu
        -155                -160
```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 166 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein, C-terminus of rnhB ex *L. lactis* MG1363

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```
Ala Val Ile Leu Pro Lys Asn Cys Lys Ile Arg Gly Leu Asn Asp Ser
                5                   10                  15

Lys Lys Val Pro Lys Ser Lys His His Ala Ile Leu Ser Glu Ile Gln
            20                  25                  30

Glu Lys Ala Leu Ala Ile Gly Val Gly Ile Val Asp Ala Glu Lys Ile
        35                  40                  45

Asp Glu Val Asn Ile Tyr Glu Ala Thr Lys Ile Ala Met Ile Gln Ala
    50                  55                  60

Val Ser Lys Leu Ser Leu Lys Pro Glu His Leu Leu Ile Asp Ala Met
65                  70                  75                  80

Val Leu Asp Leu Pro Ile Ala Gln Thr Lys Ile Ile His Gly Asp Ala
                85                  90                  95
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Arg Ser Ala Ser Ile Ala Ala Ala Ser Ile Val Ala Lys Val Thr Arg
            100                 105                 110

Asp Glu Met Met Lys Asp Phe Ala Leu Glu Phe Pro Glu Tyr Asp Phe
        115                 120                 125

Glu His Asn Ala Gly Tyr Gly Thr Ala Lys His Leu Ala Ala Leu Thr
    130                 135                 140

Lys Tyr Gly Ile Thr Arg Ile His Arg Lys Ser Tyr Glu Pro Ile Lys
145                 150                 155                 160

Ser Met Val Asn Phe Lys
                165
```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 276 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein, rggL = gadR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```
Met Tyr Lys Lys Tyr Gly Asp Cys Phe Lys Lys Leu Arg Asn Gln Lys
                5                   10                  15

Asn Leu Gly Leu Ser Tyr Phe Ser Lys Leu Gly Ile Asp Arg Ser Asn
            20                  25                  30

Ile Ser Arg Phe Glu His Gly Lys Cys Met Met Ser Phe Glu Arg Ile
        35                  40                  45

Asp Leu Met Leu Glu Glu Met Gln Val Pro Leu Ser Glu Tyr Glu Leu
    50                  55                  60

Ile Val Asn Asn Phe Met Pro Asn Phe Gln Glu Phe Phe Ile Leu Glu
65                  70                  75                  80

Leu Glu Lys Ala Glu Phe Ser Gln Asn Arg Asp Lys Ile Lys Glu Leu
                85                  90                  95

Tyr Ser Glu Val Lys Glu Thr Gly Asn His Leu Leu Thr Val Thr Val
            100                 105                 110

Lys Thr Lys Leu Gly Asn Ile Ser Gln Thr Glu Val Lys Glu Ile Glu
        115                 120                 125

Ala Tyr Leu Cys Asn Ile Glu Glu Trp Gly Tyr Phe Glu Leu Thr Leu
    130                 135                 140

Phe Tyr Phe Val Ser Asp Tyr Leu Asn Val Asn Gln Leu Glu Leu Leu
145                 150                 155                 160

Leu Phe Asn Phe Asp Lys Arg Cys Glu Asn Tyr Cys Arg Val Leu Lys
                165                 170                 175

Tyr Arg Arg Arg Leu Leu Gln Ile Ala Tyr Lys Ser Val Ala Ile Tyr
            180                 185                 190

Ala Ala Lys Gly Glu Arg Lys Lys Ala Glu Asn Ile Leu Glu Met Thr
        195                 200                 205

Lys Lys Tyr Arg Thr Val Gly Val Asp Leu Tyr Ser Glu Val Leu Arg
    210                 215                 220
```

His Leu Ala Arg Ala Ile Ile Ile Phe Asn Phe Glu Asn Ala Glu Ile
225 230 235 240

Gly Glu Glu Lys Ile Asn Tyr Ala Leu Glu Ile Leu Glu Glu Phe Gly
245 250 255

Gly Lys Lys Ile Lys Glu Phe Tyr Gln Asn Lys Met Glu Lys Tyr Leu
260 265 270

Lys Arg Ser Ile ***
275

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 503 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein, orfX = gadC

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Met Asn Gln Lys Lys Leu Ser Leu Phe Gly Phe Phe Ala Leu Thr Ala
5 10 15

Ser Met Val Leu Thr Val Tyr Glu Tyr Pro Thr Phe Ala Thr Ser Lys
20 25 30

Leu His Leu Val Phe Phe Leu Leu Leu Gly Gly Leu Leu Trp Phe Leu
35 40 45

Pro Val Ala Leu Cys Ala Ala Glu Met Ala Thr Val Glu Gly Trp Lys
50 55 60

Asn Gly Gly Ile Phe Ser Trp Val Ser Gln Thr Leu Gly Glu Arg Phe
65 70 75 80

Gly Phe Ala Ala Ile Phe Phe Gln Trp Phe Gln Ile Thr Val Gly Phe
85 90 95

Val Thr Met Ile Tyr Phe Ile Leu Gly Ala Leu Ser Tyr Val Leu Asn
100 105 110

Phe Gln Ala Leu Asn Thr Asp Pro Leu Ile Lys Phe Ile Gly Leu Leu
115 120 125

Ile Ile Phe Trp Gly Leu Thr Phe Ser Gln Leu Gly Gly Thr Gln Arg
130 135 140

Thr Ala Lys Leu Val Lys Ala Gly Phe Val Val Gly Ile Val Ile Pro
145 150 155 160

Ser Val Ile Leu Phe Gly Leu Ala Ala Ala Tyr Phe Ile Gly Gly Asn
165 170 175

Pro Ile Glu Ile Pro Ile Asn Ser His Ala Phe Val Pro Asp Phe Ser
180 185 190

Gln Val Ser Thr Leu Val Val Phe Val Ser Phe Ile Leu Ala Tyr Met
195 200 205

Gly Val Glu Ala Ser Ala Ser His Ile Asn Glu Leu Glu Asn Pro Lys
210 215 220

Arg Asn Tyr Pro Leu Ala Met Ile Leu Leu Val Ile Leu Ala Ile Ser
225 230 235 240

Leu Asp Ala Ile Gly Gly Phe Ser Val Ala Ala Val Ile Pro Gln Lys
245 250 255

Glu Leu Ser Leu Ser Ala Gly Val Ile Gln Thr Phe Gln Thr Leu Ile
260 265 270

Leu His Phe Asn His His Leu Gly Trp Leu Val Lys Val Ile Ala Leu
275 280 285

Met Ile Ala Phe Gly Val Met Gly Glu Val Ser Ser Trp Val Val Gly
290 295 300

Pro Ser Arg Gly Met Phe Ala Ala Ala Gln Arg Gly Leu Leu Pro Lys
305 310 315 320

Phe Leu Arg Lys Thr Asn Thr His Glu Val Pro Val Pro Leu Val Met
325 330 335

Ile Gln Gly Ile Ile Val Thr Leu Trp Gly Ala Val Leu Thr Phe Gly
340 345 350

Gly Gly Gly Asn Asn Leu Ser Phe Leu Val Ala Ile Ser Leu Thr Val
355 360 365

Val Ile Tyr Leu Val Gly Tyr Leu Leu Phe Phe Ile Val Tyr Phe Val
370 375 380

Leu Ile Tyr Lys Lys Gln Asn Leu Lys Arg Thr Tyr Asn Val Pro Gly
385 390 395 400

Lys Ile Ile Gly Lys Thr Ile Ile Ala Gly Ile Gly Phe Leu Leu Ser
405 410 415

Ile Phe Ala Leu Phe Ile Ser Phe Val Pro Pro Ala Ser Ile Ala Lys
420 425 430

Asn Glu Thr His Thr Tyr Gln Met Ile Leu Leu Ile Ser Phe Val Val
435 440 445

Thr Ala Ile Leu Pro Phe Ile Ile Tyr Glu Leu His Asp Lys Lys Gly
450 455 460

His Asp Thr Ile Glu Glu Pro Thr His Phe Lys Ala Gly Asp Val Asn
465 470 475 480

Pro Ala Ile Tyr Pro Ala Ala Arg Gly Glu His His Ile Ile Lys Lys
485 490 495

Glu Glu His Ile Leu Lys His
500 503

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 466 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein, gadB

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Met Leu Tyr Gly Lys Glu Asn Arg Asp Glu Ala Glu Phe Leu Glu Pro
5 10 15

Ile Phe Gly Ser Glu Ser Glu Gln Val Asp Leu Pro Lys Tyr Lys Leu
20 25 30

Ala Gln Gln Ser Ile Glu Pro Arg Val Ala Tyr Gln Leu Val Gln Asp
35 40 45

Glu Met Leu Asp Glu Gly Asn Ala Arg Leu Asn Leu Ala Thr Phe Cys
50 55 60

Gln Thr Tyr Met Glu Pro Glu Ala Val Lys Leu Met Ser Gln Thr Leu
65 70 75 80

Glu Lys Asn Ala Ile Asp Lys Ser Glu Tyr Pro Arg Thr Thr Glu Ile
85 90 95

Glu Asn Arg Cys Val Asn Met Ile Ala Asp Leu Trp Asn Ala Ser Glu
100 105 110

Lys Gly Lys Ile Tyr Gly Thr Ser Thr Ile Gly Ser Ser Glu Ala Cys
115 120 125

Met Leu Gly Gly Met Ala Met Lys Phe Ser Trp Arg Lys Arg Ala Glu
130 135 140

Lys Leu Gly Leu Asp Ile Asn Ala Lys Lys Pro Asn Leu Val Ile Ser
145 150 155 160

Ser Gly Tyr Gln Val Cys Trp Glu Lys Phe Cys Val Tyr Trp Asp Ile
165 170 175

Glu Met Arg Glu Val Pro Met Asp Arg Glu His Met Ser Ile Asn Leu
180 185 190

Glu Lys Val Met Asp Tyr Val Asp Glu Tyr Thr Ile Gly Val Val Gly
195 200 205

Ile Met Gly Ile Thr Tyr Thr Gly Arg Tyr Asp Asp Ile Lys Ala Leu
210 215 220

Asp Asn Leu Ile Glu Glu Tyr Asn Lys Gln Thr Asp Tyr Lys Val Tyr
225 230 235 240

Ile His Val Asp Ala Ala Ser Gly Gly Leu Tyr Ala Pro Phe Val Glu
245 250 255

Pro Glu Leu Glu Trp Asp Phe Arg Leu Lys Asn Val Ile Ser Ile Asn
260 265 270

Thr Ser Gly His Lys Tyr Gly Leu Val Tyr Pro Gly Val Gly Trp Val
275 280 285

Leu Trp Arg Asp Lys Lys Tyr Leu Pro Glu Glu Leu Ile Phe Lys Val
290 295 300

Ser Tyr Leu Gly Gly Glu Leu Pro Thr Met Ala Ile Asn Phe Ser His
305 310 315 320

Ser Ala Ser Gln Leu Ile Gly Gln Tyr Tyr Asn Phe Val Arg Tyr Gly
325 330 335

Phe Asp Gly Tyr Lys Ala Ile His Glu Arg Thr His Lys Val Ala Met
340 345 350

Tyr Leu Ala Glu Glu Ile Glu Lys Thr Gly Met Phe Glu Ile Met Asn
355 360 365

Asp Gly Ala Gln Leu Pro Ile Val Cys Tyr Lys Leu Lys Glu Asn Ser
370 375 380

Asn Arg Gly Trp Asn Leu Tyr Asp Leu Ala Asp Arg Leu Leu Met Lys
385 390 395 400

```
Gly Trp Gln Val Pro Ala Tyr Pro Leu Pro Lys Asn Leu Glu Asn Glu
                405                 410                 415

Ile Ile Gln Arg Leu Val Ile Arg Ala Asp Phe Gly Met Asn Met Ala
            420                 425                 430

Phe Asn Tyr Val Gln Asp Met Gln Glu Ala Ile Asp Ala Leu Asn Lys
        435                 440                 445

Ala His Ile Leu Phe His Gln Glu Pro Glu Asn Lys Thr Tyr Gly Phe
    450                 455                 460

Thr His
465
```

# CLAIMS

1. A salt-inducible promoter **derivable from a lactic acid bacterium**, in isolation from the coding sequence which is normally controlled by said promoter in a wild-type lactic acid bacterium.

2. An essential part of a salt-inducible promoter according to claim 1, which comprises the polynucleotide 1482-1925 of SEQ. ID. NO: 10.

3. An essential part according to claim 2, which comprises the polynucleotide 1482-2068 of SEQ. ID. NO: 10.

4. A salt-inducible promoter according to claim 1, which comprises the polynucleotide 1-2068 of SEQ. ID. NO: 10.

5. A salt-inducible promoter according to claim 4, which additionally comprises part of the ORF X gene together forming polynucleotide 1-2426 of SEQ. ID. NO: 10.

6. A modification of a salt-inducible promoter according to claim 1 or an essential part thereof, which comprises a DNA sequence essentially corresponding to a polynucleotide selected from the group consisting of
(a) polynucleotide 1482-1925 of SEQ. ID. NO: 10 followed by a promoter fuctional in a lactic acid bacterium,
(b) polynucleotide 1482-2068 of SEQ. ID. NO: 10,
(c) polynucleotide 1-2068 of SEQ. ID. NO: 10, and
(d) polynucleotide 1-2426 of SEQ. ID. NO: 10.

7. A DNA fragment capable of regulating a salt-inducible promoter active in a lactic acid bacterium, which comprises the polynucleotide 1095-1925 of SEQ. ID. NO: 10, or a modification thereof that
(a) encodes the same polypeptide as said polynucleotide 1095-1925, or

(b) encodes a modification of such polypeptide still having essentially the same regulating capacity.

8. A recombinant vector comprising a salt-inducible promoter or an essential part thereof as claimed in any one of claims 1-6, or a DNA fragment as claimed in claim 7 in combination with a DNA fragment selected from the group consisting of a DNA fragment containing the polynucleotide 1926-2000 of SEQ. ID. NO: 10 and modifications thereof still having essentially the same promoting capacity.

9. A transformed lactic acid bacterium comprising a salt-inducible promoter or an essential part thereof as claimed in any one of claims 1-6, or a DNA fragment as claimed in claim 7 in combination with a DNA fragment selected from the group consisting of a DNA fragment containing the polynucleotide 1926-2000 of SEQ. ID. NO: 10 and modifications thereof still having essentially the same promoting capacity.

10. A process for the production of a desired protein by a transformed lactic acid bacterium, whereby the gene encoding said desired protein or a precursor thereof is expressed under control of an inducible promoter, **characterised in that** the promoter is a salt-inducible promoter or an essential part thereof as claimed in any one of claims 1-6, or a DNA fragment as claimed in claim 7 in combination with a DNA fragment selected from the group consisting of a DNA fragment containing the polynucleotide 1926-2000 of SEQ. ID. NO: 10 and modifications thereof still having essentially the same promoting capacity.

11. A process according to claim 10, in which the desired protein is secreted by the lactic acid bacterium due to the presence of a DNA fragment fused to the gene encoding the desired protein and effecting secretion of the desired protein or a precursor thereof.

12. A process according to claim 10, in which the action of the salt-inducible promoter is enhanced by allowing the pH of the medium in which the lactic acid bacterium is cultured to decrease to a value of about 4 to about 4.5.

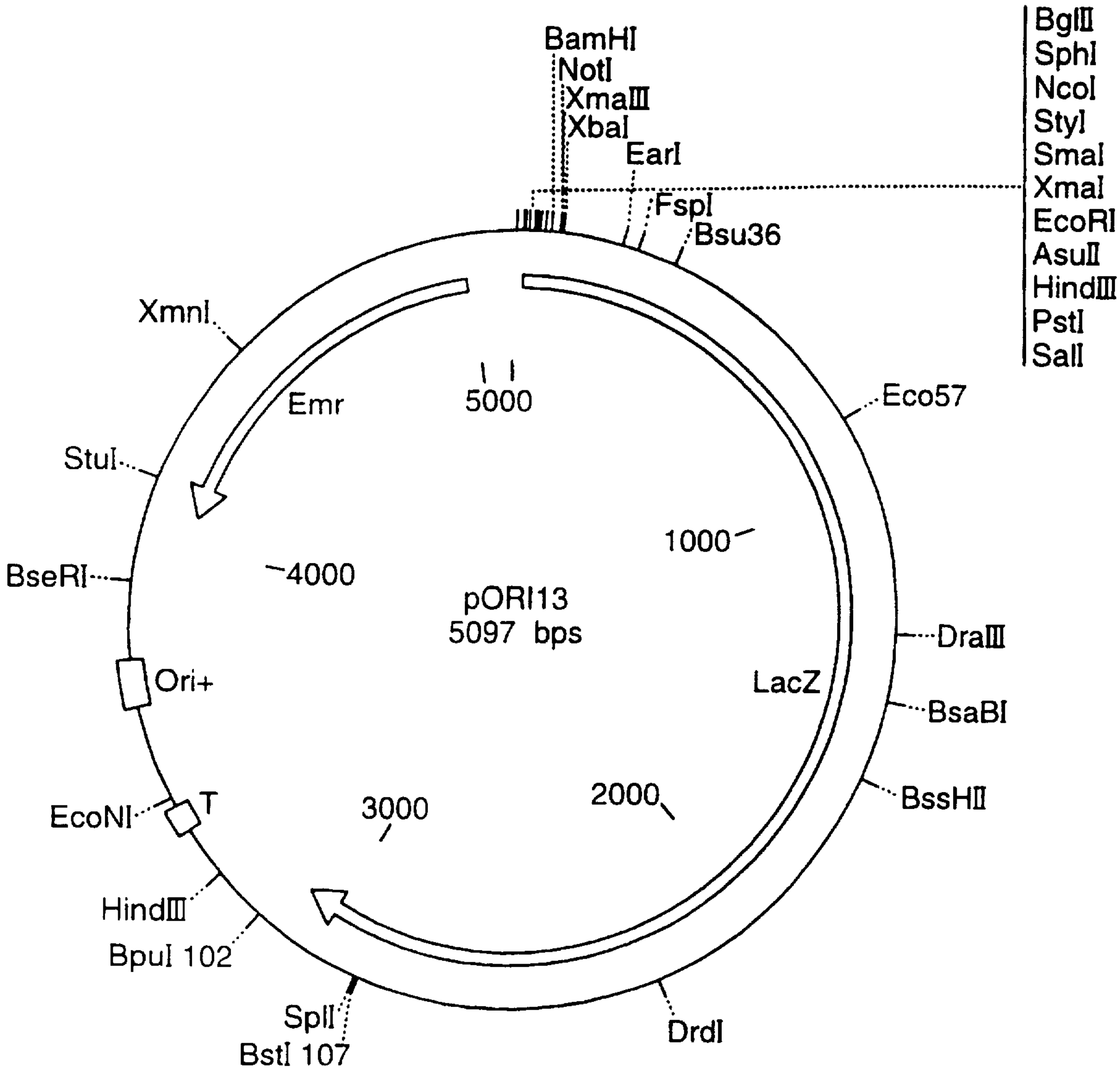
13. A process according to anyone of claims 10-12, in which the action of the salt-inducible promoter is enhanced by incorporating a sufficient amount of glutamate or glutamic acid, preferably about 50 mM, in the medium in which the lactic acid bacterium is cultured.

14. Use of a process as claimed in any one of claims 10-13 in a fermentation process, in which the desired protein is a lytic protein causing lysis of the bacterial cells so that the contents of the cells can be released.

15. Use of a process as claimed in any one of claims 10-13 in a fermentation process, in which the desired protein is an enzyme involved in flavour formation.

16. Use of a process as claimed in any one of claims 10-13 in a fermentation process, in which the desired protein is a protein having a function in a cheese production process, such as chymosin or a precursor thereof, or an enzyme involved in cheese flavour formation.

Fig.1.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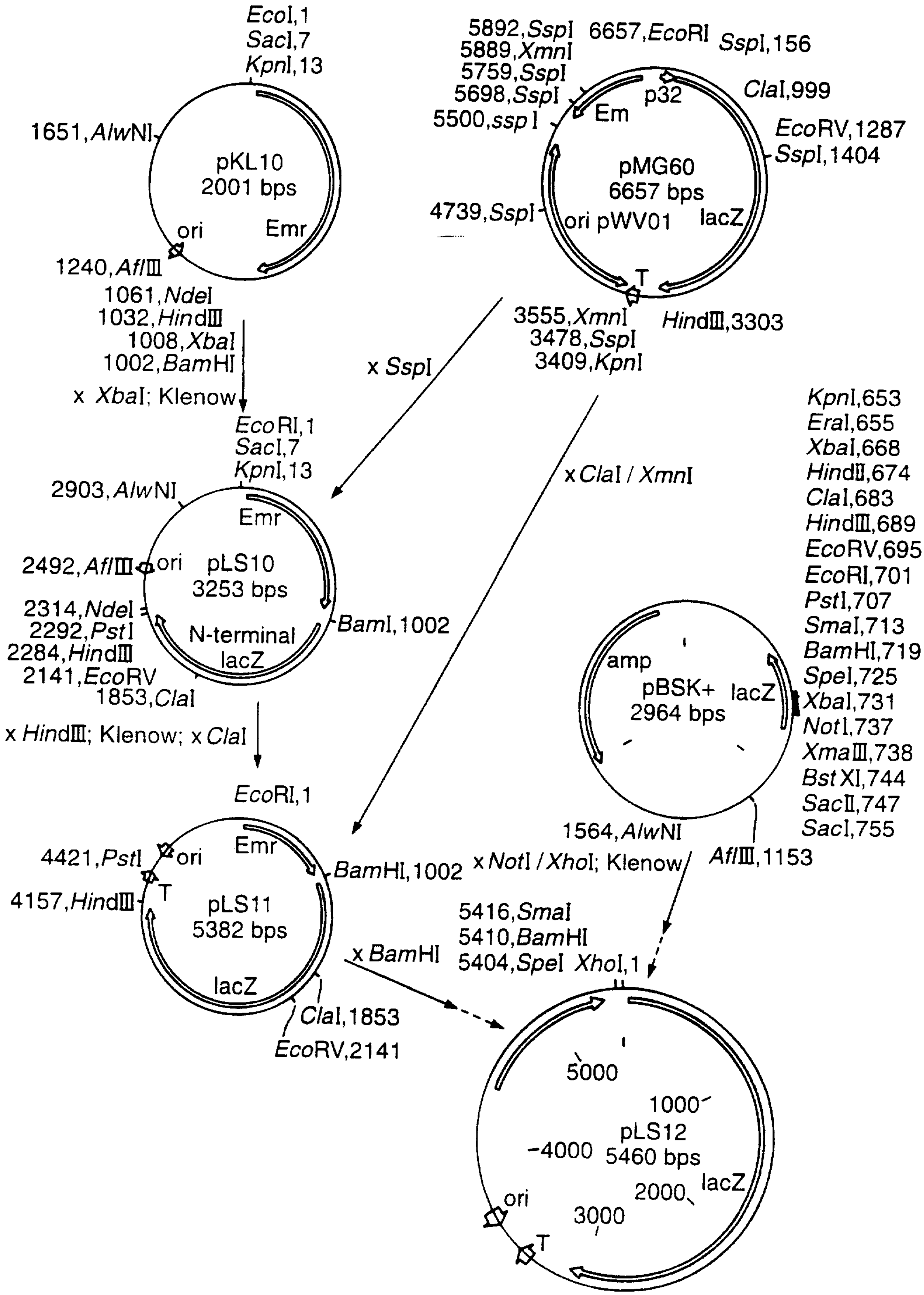
XmaIII
XbaI
EarI
FspI
Bsu36
BglII
SphI
NcoI
StyI
SmaI
XmaI
EcoRI
AsuII
HindIII
PstI
SalI
Eco57
DraIII
BsaBI
BssHII
DrdI
SplI
BstI 107
BpuI 102
HindIII
EcoNI
T
Ori+
BseRI
StuI
XmnI
Emr
5000
4000
1000
pORI13
5097 bps
LacZ
3000
2000


XbaI
RBS
Met Lys Gly
...MCS...TCTAGATTCGGAGGAATTTTGAA ATG AAA GGG.. lacZ
*** stop
*** stop
** * stop

# Fig.2.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KpnI,13
1651,AlwNI
pKL10
2001 bps
ori
Emr
1240,AflIII
1061,NdeI
1032,HindIII
1008,XbaI
1002,BamHI
x XbaI; Klenow
5892,SspI
5889,XmnI
5759,SspI
5698,SspI
5500,ssp I
6657,EcoRI
SspI,156
ClaI,999
EcoRV,1287
SspI,1404
Em
p32
pMG60
6657 bps
4739,SspI
ori pWV01
lacZ
T
3555,XmnI
3478,SspI
3409,KpnI
HindIII,3303
x SspI
x ClaI / XmnI
EcoRI,1
SacI,7
KpnI,13
2903,AlwNI
Emr
2492,AflIII
ori
pLS10
3253 bps
2314,NdeI
2292,PstI
2284,HindIII
2141,EcoRV
1853,ClaI
N-terminal
lacZ
BamI,1002
x HindIII; Klenow; x ClaI
KpnI,653
EraI,655
XbaI,668
HindII,674
ClaI,683
HindIII,689
EcoRV,695
EcoRI,701
PstI,707
SmaI,713
BamHI,719
SpeI,725
XbaI,731
NotI,737
XmaIII,738
BstXI,744
SacII,747
SacI,755
amp
pBSK+
2964 bps
lacZ
1564,AlwNI
AflIII,1153
EcoRI,1
4421,PstI
ori
Emr
BamHI,1002
4157,HindIII
T
pLS11
5382 bps
lacZ
ClaI,1853
EcoRV,2141
x NotI / XhoI; Klenow
x BamHI
5416,SmaI
5410,BamHI
5404,SpeI
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1000
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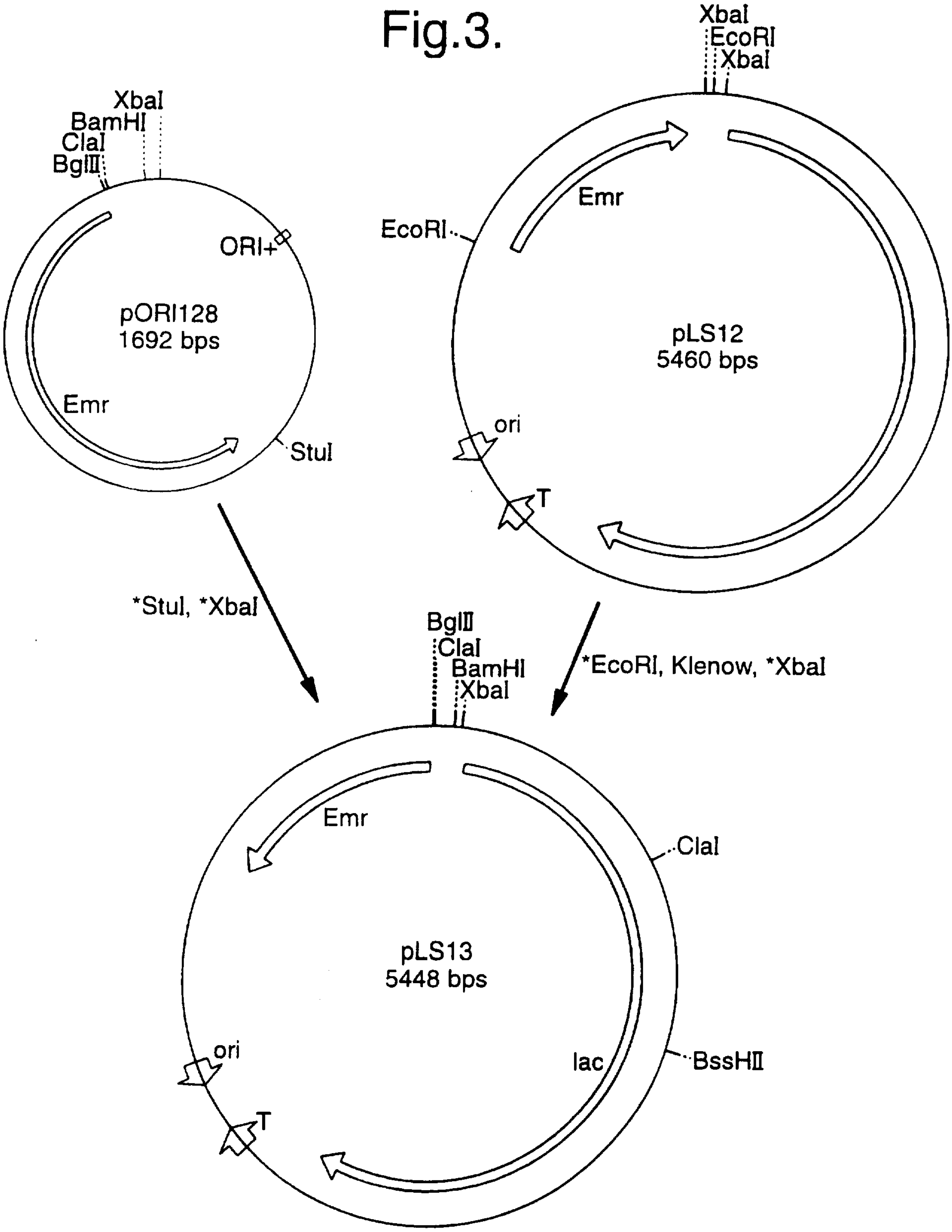
5460 bps
4000
lacZ
2000
ori
3000
T

Fig.3.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ClaI
BglII
ORI+
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Emr
StuI
XbaI
EcoRI
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Emr
EcoRI
pLS12
5460 bps
ori
T
*StuI, *XbaI
*EcoRI, Klenow, *XbaI
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lac
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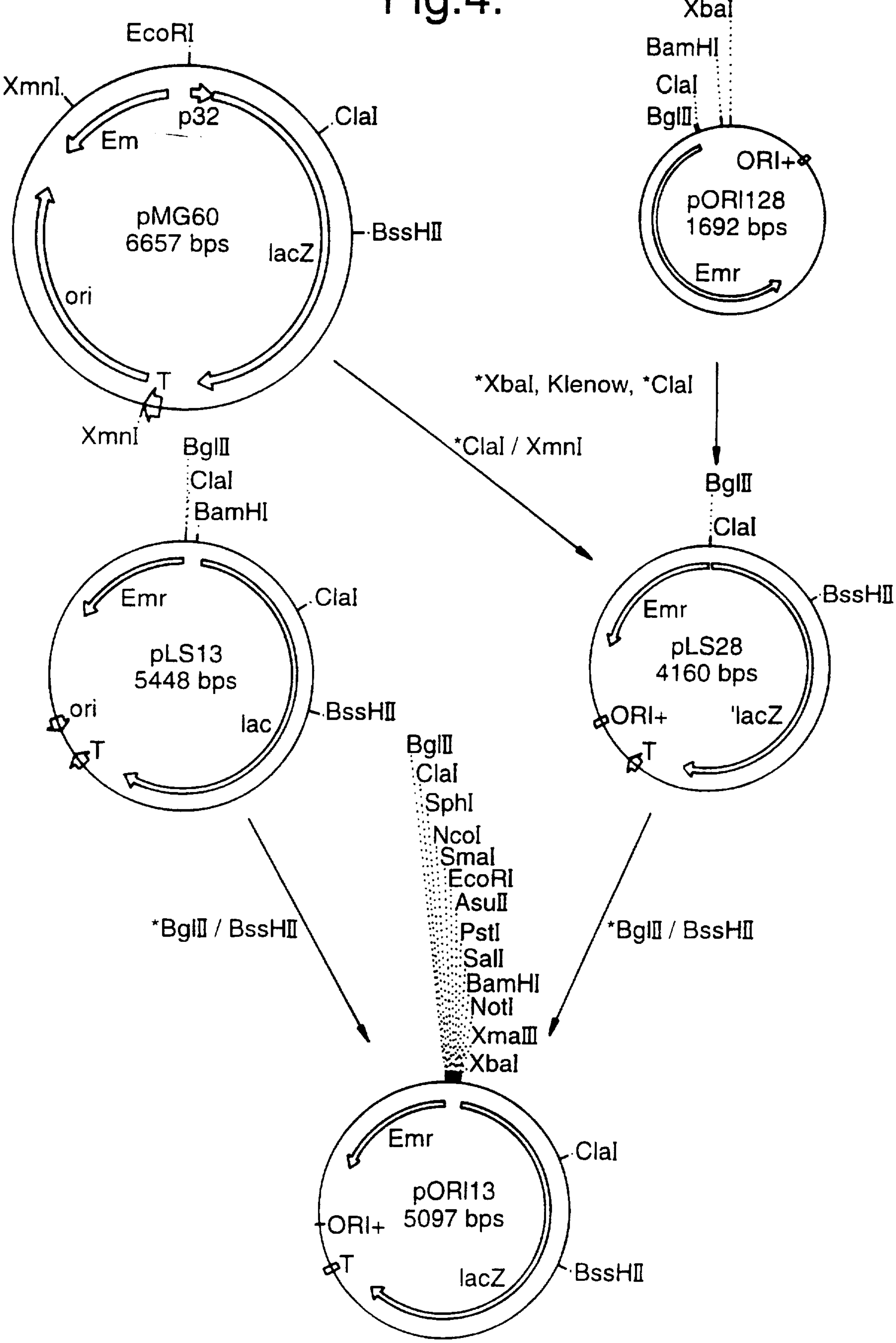
Fig.4.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T
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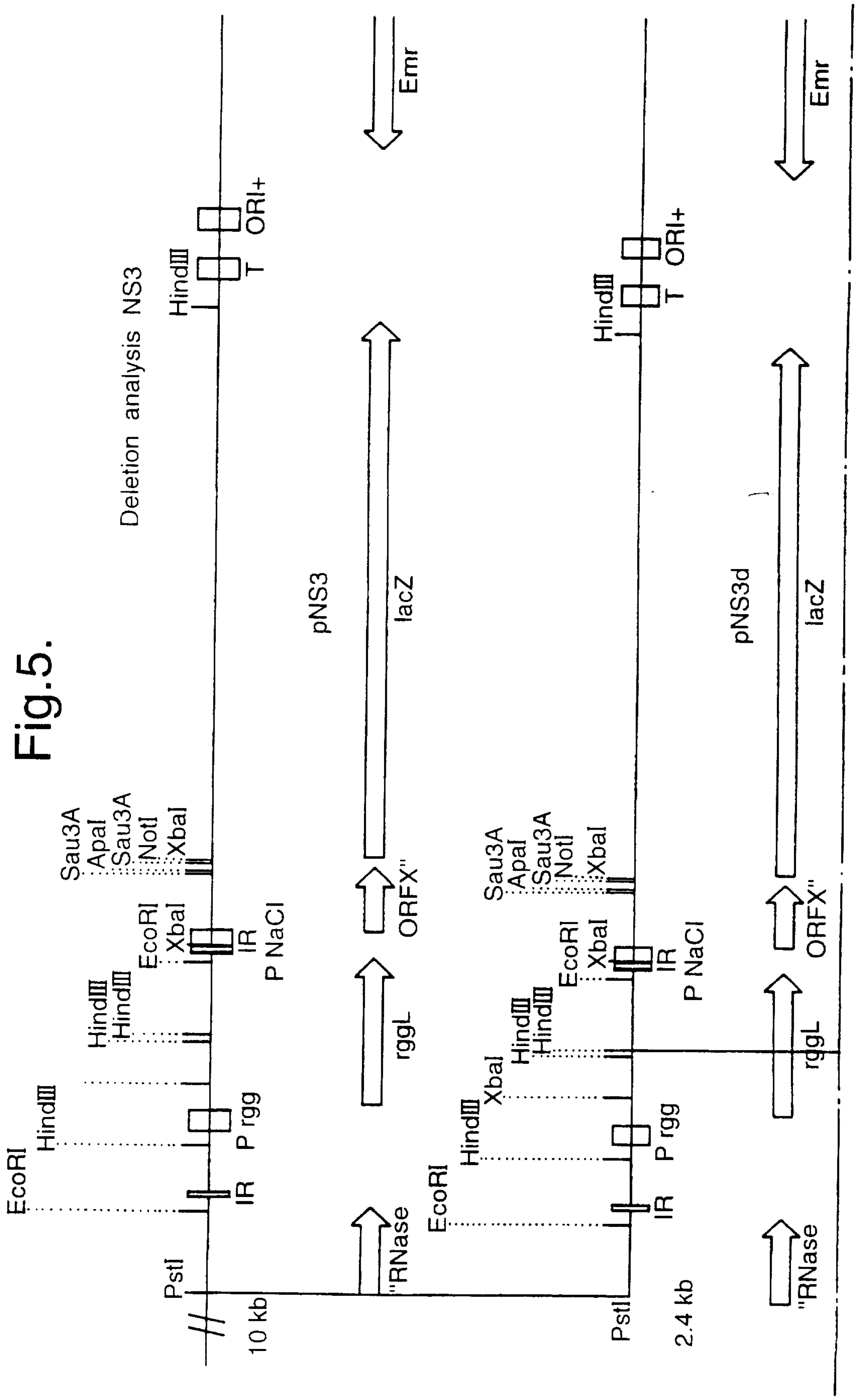
ClaI
BglII
ORI+
pORI128
1692 bps
Emr
*XbaI, Klenow, *ClaI
*ClaI / XmnI
BglII
ClaI
BamHI
Emr
ClaI
pLS13
5448 bps
ori
BssHII
lac
T
BglII
ClaI
Emr
BssHII
pLS28
4160 bps
ORI+
'lacZ
T
BglII
ClaI
SphI
NcoI
SmaI
EcoRI
AsuII
PstI
SalI
BamHI
NotI
XmaIII
XbaI
*BglII / BssHII
*BglII / BssHII
Emr
ClaI
pORI13
5097 bps
ORI+
T
lacZ
BssHII


Deletion analysis NS3
pNS3
PstI
EcoRI
HindIII
HindIII
HindIII
EcoRI
XbaI
Sau3A
ApaI
Sau3A
NotI
XbaI
HindIII
IR
P rgg
IR
P NaCl
T
ORI+
10 kb
"RNase
rggL
ORFX"
lacZ
Emr
pNS3d
PstI
2.4 kb
EcoRI
HindIII
XbaI
HindIII
HindIII
EcoRI
XbaI
Sau3A
ApaI
Sau3A
NotI
XbaI
HindIII
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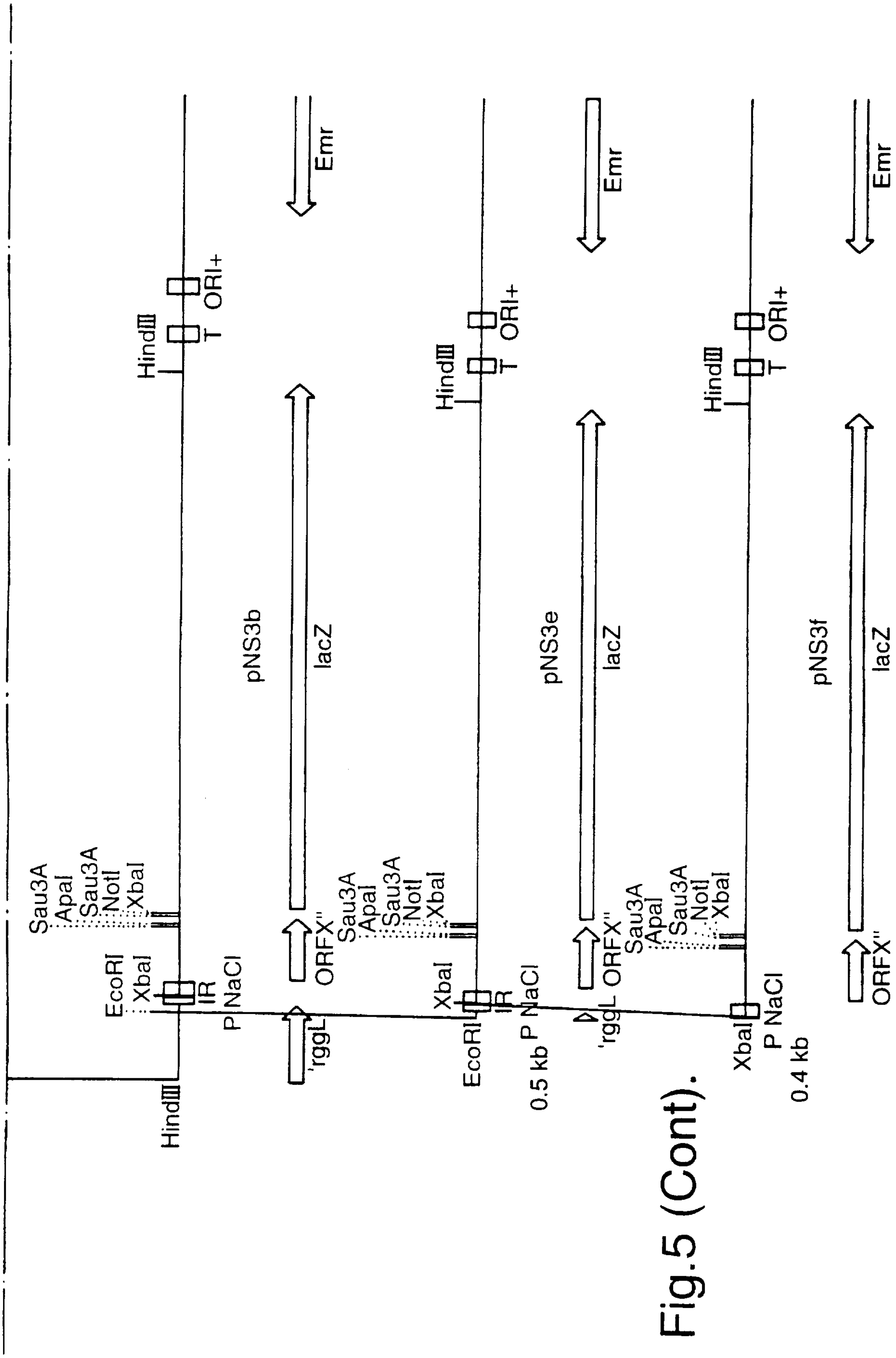
P rgg
IR
P NaCl
T
ORI+
"RNase
rggL
ORFX"
lacZ
Emr

Fig.5.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IR
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HindIII
T
ORI+
'rggL
ORFX''
lacZ
Emr
pNS3b
XbaI
EcoRI
0.5 kb
pNS3e
XbaI
0.4 kb
pNS3f

Fig.5 (Cont).

# Fig.6. (1/3)

```
        PstI    'rnhB
   1   CTGCAGTAAT TTTGCCAAAG AATTGTAAAA TTCGTGGTTT GAATGATAGT AAAAAAGTGC
   1     AlaVal IleLeuProLys AsnCysLys IleArgGly LeuAsnAspSer LysLysVal

  61   CAAAATCAAA GCATCATGCT ATTCTATCTG AAATTCAAGA AAAAGCGCTA GCGATTGGAG
  20  ProLysSer LysHisHisAla IleLeuSer GluIleGln GluLysAlaLeu AlaIleGly

 121   TTGGAATTGT TGATGCCGAA AAAATTGATG AAGTAAATAT TTATGAGGCG ACAAAAATTG
  40  ValGlyIle ValAspAlaGlu LysIleAsp GluValAsn IleTyrGluAla ThrLysIle

 181   CAATGATTCA GGCAGTATCA AAATTATCTC TTAAACCTGA ACATCTCTTA ATAGATGCGA
  60  AlaMetIle GlnAlaValSer LysLeuSer LeuLysPro GluHisLeuLeu IleAspAla

 241   TGGTTTTAGA TTTGCCCATT GCTCAGACGA AAATCATTCA TGGAGATGCT CGTTCAGCTT
  80  MetValLeu AspLeuProIle AlaGlnThr LysIleIle HisGlyAspAla ArgSerAla

         NotI
 301   CAATTGCGGC CGCATCAATT GTAGCTAAAG TGACTCGTGA TGAAATGATG AAGGATTTCG
 100  SerIleAla AlaAlaSerIle ValAlaLys ValThrArg AspGluMetMet LysAspPhe

 361   CTTTAGAATT TCCAGAATAT GATTTTGAAC ATAATGCAGG CTATGGAACA GCAAAACATC
 120  AlaLeuGlu PheProGluTyr AspPheGlu HisAsnAla GlyTyrGlyThr AlaLysHis

                                         EcoRI
 421   TTGCAGCTCT GACAAAATAT GGTATCACAA GAATTCATCG GAAATCCTAT GAACCAATTA
 140  LeuAlaAla LeuThrLysTyr GlyIleThr ArgIleHis ArgLysSerTyr GluProIle

 481   AATCGATGGT CAATTTCAAA TAGTAGATTA TGTAAGTAAA AAAAGGAAAA CGTGAACGAT
 160  LysSerMet ValAsnPheLys ***                               ---- ----- - >

 541   ATTTGGTCAC GTTTTTTTGC TGACAAGTCT GTCAGTAATT ATTTTCAAAG GTTTCAAAAA
         < - ---- ------

 601   TATAGTCTAG TAATTTGCTA GACTAATCTT CTGTTTTTTA ATAATAAACT AATTTTTTGT

 661   TAATCTAAAT GACAAAATTA ATAAGCAGAG TTTTTTATAA AATTAGCTAC TTATAAAAAA

 721   TTTGAAATTG GTATAGTTAA ATCTGTTATA ATTTCCAATA TTTTTTAATA ATAATTATTT

                                                              HindIII
 781   TAACAAAATA CTTATATCAA AACTCTTTCA AAGTATATAA TGAGCGTTGT ATAAGCTTTT

 841   ATGTCTTTCT ATATCAACTT TTAATAGAAA TATAAAGTAA TATAAATGTT TTTATAATAA

                                                   -35
 901   ATTATGTGAG ATATATTTTT TTGTCCGTAC TGGTATAGAT TTGACGATTA AGTCTTAAAT

         -10
 961   AAGTTATAAT CTCAATTGCG TAATTTCTTA AATACAGAAA TAACAACTAC ATTGGTAGAC
                        ↑

1021   TGATTAAAAA GTGTACTTGA TGAACTGTTA TAAACCTTAA AAAAATAAAA ATAATAGTTT

                    start rggL
1081   GGGggaTgtT AAAGATGTAT AAAAAATATG GAGATTGTTT TAAAAAGTTG CGAAACCAAA
   1       rbs         MetTyr LysLysTyr GlyAspCys PheLysLysLeu ArgAsnGln

                                                                XbaI
1141   AGAATTTAGG GTTATCATAC TTTAGTAAAC TTGGAATAGA CCGTTCAAAT ATATCTAGAT
  16  LysAsnLeu GlyLeuSerTyr PheSerLys LeuGlyIle AspArgSerAsn IleSerArg

1201   TTGAACATGG AAAATGTATG ATGAGTTTTG AGCGTATAGA TTTGATGTTA GAAGAAATGC
  36  PheGluHis GlyLysCysMet MetSerPhe GluArgIle AspLeuMetLeu GluGluMet
```

# Fig.6. (2/3)

```
1261  AAGTTCCGTT ATCTGAGTAC GAATTGATTG TAAATAATTT TATGCCGAAT TTCCAAGAAT
  56 GlnValPro LeuSerGluTyr GluLeuIle ValAsnAsn PheMetProAsn PheGlnGlu

1321  TTTTTATATT AGAATTGGAA AAAGCTGAAT TTAGCCAAAA TCGAGATAAA ATAAAAGAGT
  76 PhePheIle LeuGluLeuGlu LysAlaGlu PheSerGln AsnArgAspLys IleLysGlu

                                                              HindIII
1381  TGTATTCTGA GGTCAAAGAA ACGGGGAATC ATTTACTGAC GGTTACCGTG AAAACGAAGC
  96 LeuTyrSer GluValLysGlu ThrGlyAsn HisLeuLeu ThrValThrVal LysThrLys

                                          HindIII
1441  TTGGGAATAT AAGTCAGACA GAAGTTAAAG AAATTGAAGC TTATCTTTGC AATATTGAAG
 116 LeuGlyAsn IleSerGlnThr GluValLys GluIleGlu AlaTyrLeuCys AsnIleGlu

1501  AGTGGGGATA TTTTGAACTT ACTTTATTTT ATTTTGTATC TGATTATCTC AATGTCAATC
 136 GluTrpGly TyrPheGluLeu ThrLeuPhe TyrPheVal SerAspTyrLeu AsnValAsn

1561  AATTAGAATT GCTGCTTTTT AATTTTGATA AAAGATGTGA AAATTACTGT AGAGTCTTAA
 156 GlnLeuGlu LeuLeuLeuPhe AsnPheAsp LysArgCys GluAsnTyrCys ArgValLeu

1621  AATATAGAAG GAGACTATTG CAAATAGCCT ATAAAAGTGT TGCGATATAC GCGGCTAAAG
 176 LysTyrArg ArgArgLeuLeu GlnIleAla TyrLysSer ValAlaIleTyr AlaAlaLys

1681  GAGAAAGAAA AAAAGCCGAA AATATTTTAG AAATGACTAA AAAATATCGA ACTGTGGGAG
 196 GlyGluArg LysLysAlaGlu AsnIleLeu GluMetThr LysLysTyrArg ThrValGly

1741  TCGATTTATA TTCAGAAGTA TTAAGACATC TTGCTAGAGC TATCATTATT TTTAATTTTG
 216 ValAspLeu TyrSerGluVal LeuArgHis LeuAlaArg AlaIleIleIle PheAsnPhe

1801  AAAATGCAGA GATTGGGGAA GAAAAAATAA ATTATGCTCT TGAGATTTTG GAAGAATTTG
 236 GluAsnAla GluIleGlyGlu GluLysIle AsnTyrAla LeuGluIleLeu GluGluPhe

                        EcoRI
1861  GAGGAAAGAA GATAAAAGAA TTCTATCAGA ATAAAATGGA AAAGTATTTG AAAAGGTCAA
 256 GlyGlyLys LysIleLysGlu PheTyrGln AsnLysMet GluLysTyrLeu LysArgSer

1921  TTTAGTCTCT TTTGAGCTGT TGCTTTAAAG CAACAGCTCA AAAGAGATTT TCTTTATTCT
 276 Ile***---------------------><--------------------

     XbaI      -10
1981  AGAGCATATA CTAGAGGGTG AAGATAGGTT GTCTGAAGCA TTATAACTTG TCTTTTAAAA

                                 Start orfX
2041  AATTCAATCA TAAATATaag gaggtATGAT GAATCAAAAA AAATTATCAT TATTCGGTTT
   1                     rbs        Met AsnGlnLys LysLeuSer LeuPheGlyPhe

2101  TTTCGCATTA ACCGCTTCAA TGGTTTTGAC TGTCTATGAG TATCCGACTT TTGCCACGTC
  12   PheAlaLeu ThrAlaSer MetValLeu ThrValTyrGlu TyrProThrP heAlaThrSer

2161  AAAATTACAT TTGGTGTTCT TTTTACTTCT CGGAGGACTA CTATGGTTTT TGCCTGTAGC
  32   LysLeuHis LeuValPhe PheLeuLeu LeuGlyGlyLeu LeuTrpPhe LeuProValAla

2221  GCTCTGCGCA GCAGAAATGG CGACGGTTGA AGGCTGGAAA AATGGTGGAA TCTTTAGTTG
  52   LeuCysAla AlaGluMet AlaThrVal GluGlyTrpLys AsnGlyGly IlePheSerTrp

2281  GGTCAGTCAA ACTTTAGGTG AGCGCTTTGG TTTTGCAGCC ATATTTTTTC AGTGGTTCCA
  72   ValSerGln ThrLeuGly GluArgPhe GlyPheAlaAla IlePhePhe GlnTrpPheGln

                                                  ApaI
2341  AATTACAGTA GGTTTTGTCA CTATGATCTA TTTCATTTTA GGGGCCCTCT CTTATGTGTT
  92   IleThrVal GlyPheVal ThrMetIle TyrPheIleLeu GlyAlaLeu SerTyrValLeu
```

# Fig.6. (3/3)

```
                                  Sau3A
2401  AAATTTTCAG GCGCTCAATA CAGATCCATT GATAAAATTT ATTGGTTTAC TAATCATTTT
 112   AsnPheGln AlaLeuAsn ThrAspPro LeuIleLysPhe IleGlyLeu LeuIleIlePhe

2461  TTGGGGATTG ACTTTTTCTC AATTAGGTGG GACGCAACGG ACTGCCAAAT TAGTAAAAGC
 132   TrpGlyLeu ThrPheSer GlnLeuGly GlyThrGlnArg ThrAlaLys LeuValLysAla

2521  TGGCTTTGTA GTTGGAATAG TGATTCCATC GGTTATCTTG TTTGGATTAG CAGCGGCATA
 152   GlyPheVal ValGlyIle ValIlePro SerValIleLeu PheGlyLeu AlaAlaAlaTyr

2581  CTTTATCGGA GGCAATCCTA TAGAAATACC AATTAACAGC CATGCTTTTG TACCAGATTT
 172   PheIleGly GlyAsnPro IleGluIle ProIleAsnSer HisAlaPhe ValProAspPhe

2641  TTCACAGGTA TCAACTTTAG TAGTTTTTGT TTCTTTTATT CTGGCTTATA TGGGGGTAGA
 192   SerGlnVal SerThrLeu ValValPhe ValSerPheIle LeuAlaTyr MetGlyValGlu

2701  AGCCTCAGCT TCACATATTA ATGAACTTGA AAATCCAAAA CGAAATTATC CCTTAGCAAT
 212   AlaSerAla SerHisIle AsnGluLeu GluAsnProLys ArgAsnTyr ProLeuAlaMet

2761  GATTTTATTA GTAATTTTGG CTATTTCTTT AGATGCCATA GGTGGATTTT CTGTAGCAGC
 232   IleLeuLeu ValIleLeu AlaIleSer LeuAspAlaIle GlyGlyPhe SerValAlaAla

2821  AGTTATTCCT CAAAAAGAGT TATCATTAAG TGCAGGGGTA ATCCAAACTT TTCAAACGTT
 252   ValIlePro GlnLysGlu LeuSerLeu SerAlaGlyVal IleGlnThr PheGlnThrLeu

2881  AATCTTACAT TTTAATCATC ATTTGGGATG GTTAGTTAAA GTGATTGCAC TAATGATTGC
 272   IleLeuHis PheAsnHis HisLeuGly TrpLeuValLys ValIleAla LeuMetIleAla

                                              XbaI
2941  CTTTGGGGTT ATGGGAGAAG TGAGTTCATG GGTTGTTGGT CCTTCTAGA
 292   PheGlyVal MetGlyGlu ValSerSer TrpValValGly ProSerArg
```

# Fig.7.

```
RGGL  MY--KKYGDCFKKLRNQKNLGLSYFSKLGIDRSNISRFEHGKCMMSFERI   48
RGG1  MLIVKSSGKILKIIRESKNMSLKEVAAGDISVAQLSRYERGISSLTVDSF   50
ORF3  M-PYKRYGEIFKKLREQKNFSLHFSEIGISKASLSRFELGQTMISFERL   49
      *   *. *. .* .*. **..*.  .  .*. . .**.* *   .. ...

RGGL  DLMLEEMQVPLSEYELIVNNFMPNFQEFFILELEKAEFSQNRDKIKELYS   98
RGG1  YSCLRNMSVSLAEFQYVYHNYREADDVVLSQKLSEAQRENNIVKLESILA  100
ORF3  DSALQEMNVTLAEYEHFINNFSMDYKEEFLEDIILADIADDVDKLHKL--   97
         * .* *.*.*..   .*.  . .  .  ..  *. ...  *....

RGGL  -------EVKETGNHLL-TVTVKTKLGN------ISQTEVKEIEAYLCNI  134
RGG1  GSEAMAQEFPEKKNYKLNTIVIRATLTSCNPDYQVSKGDIEFLTDYLFSV  150
             *  *  *  * *. ... *        .*  ..  .  **  .

RGGL  EEWGYFELTLFYFVSDYLNVNQLELLLFNFDKRCENYCRVLKYRRRLLQI  184
RGG1  EEWGRYELWLFTNSVNLLTLETLETFASEMINRTQFYNNLPENRRRIIKM  200
      **** .** **      * .  **        *   *  .   ***.. .

RGGL  AYKSVAIYAAKGERKKAENILEMTKKYRTVGVDLYSEVLRHLARAIIIFN  234
RGG1  LLNVVSACIENNHLQVAMKFLNYIDNTKIPETDLYDRVLIKYHKALYSYK  250
          *.          *   *      .    ***  **    .*.  .

RGGL  FENAEIGE--EKINYALEILEEFG-GKKIKEFYQNKMEKYLKR-SI     276
RGG1  VGNPHARHDIEQCLSTFEYLDSFGVARKLKEQFERIQLTVVADLQIE    297
        *       *    . * *. **  .*.** .       .    *
```

# Fig.8.

```
L. lactis      -------------------------------AVILPKNCKIRGLNDSKKV   19
V. cholerae    ------------------------------------PNRPIMGLNDSKKL   14
E. coli        MIEFVYPHT-QLVAGVDEVGRGPLVGAVVTAAVILDPARPIAGLNDSKKL   49
H. influenzae  M--FEYPQGYKLIAGVDEVGRGPLVGAVVTAAVILDPHNPIEGLADSKKL   48
                                              ****     * **.****.

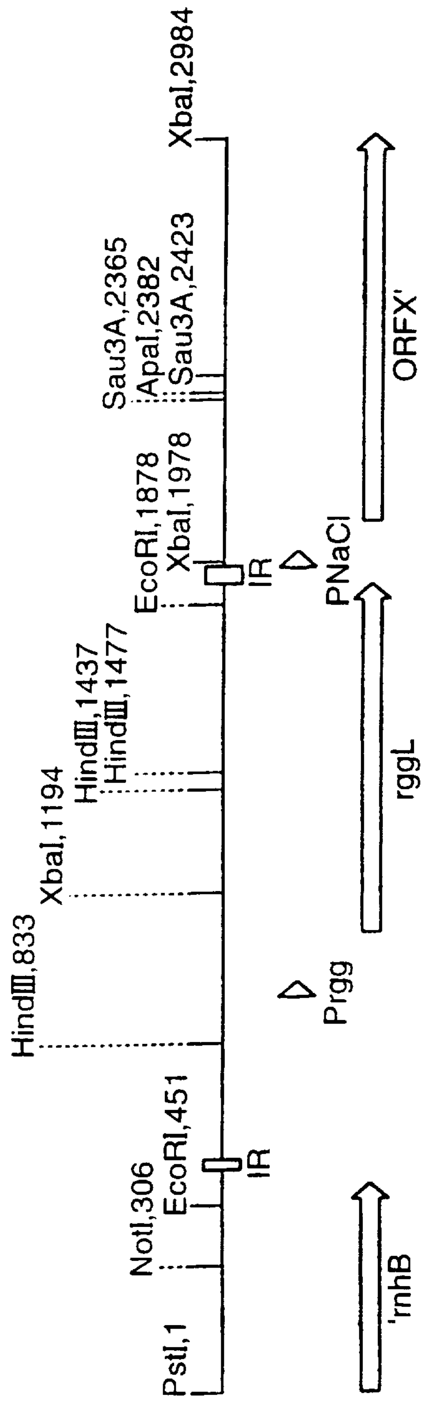
L. lactis      PKSKHHAILSEIQEKALAIGVGIVDAEKIDEVNIYEATKIAMIQAVSKLS   69
V. cholerae    SEKKRLALFPEIQVKALAWAVGRCSPQEIDELNIFQATMVAMQRAVAGLR   64
E. coli        SEKRRLALYEEIKEKALSWSLGRAEPHEIDELNILHATMLAMQRAVAGLH   99
H. influenzae  SEKKRLALAEEIKEKARAWALGRAEADEIDEINILQASLLAMTRAVKSLK   98
               ..... *.  **. **  . ..*  ....***.** .*. .** .**  *

L. lactis      LKPEHLLIDAMVL--DLPIAQTKIIHGDARSASIAAASIVAKVTRDEMMK   117
V. cholerae    IQPDLVLIDGNKIPK-LPMEAQAVVKGDLRVAQISAASIIAKVIRDQEME   113
E. coli        IAPEYVLIDGNRCPK-LPMPAMAVVKGDSRVPEISAASILAKVTRDAEMA   148
H. influenzae  IQPHFVLIDGNKIPKDLAIPAQAVVKGDSLVAEISAASILAKVARDQEME   148
               . *. .***.        *.. .  ...**   . *.****.*** **. *

L. lactis      DFALEFPEYDFEHNAGYGTAKHLAALTKYGITRIHRKSYEPIKSMVNFK-   166
V. cholerae    ALDKQYPQFGFANHKGYPTAAHFAAIEQHGVIEQHRKSFGPVKRALGE--   162
E. coli        ALDIVFPQYGFAQHKGYPTAFHLEKLAEHGATEHHRRSFGPVKRALGTCV   198
H. influenzae  ELDKQYPEYAFAQHKGYPTKLHLEKLAELGALPQHRRSFAPVKKALEQF-   197
               ...   .*...*... ** *  *.. ... *    **.*..*.*. .

L. lactis      ---------------   166
V. cholerae    ---------------   162
E. coli        LILVSRLSKPESEDV   213
H. influenzae  ---------------   197
```

Fig.9.


PstI,1
NotI,306
EcoRI,451
IR
HindIII,833
Prgg
XbaI,1194
HindIII,1437
HindIII,1477
rggL
EcoRI,1878
XbaI,1978
IR
PNaCl
Sau3A,2365
ApaI,2382
Sau3A,2423
ORFX'
XbaI,2984
'rnhB

13/34

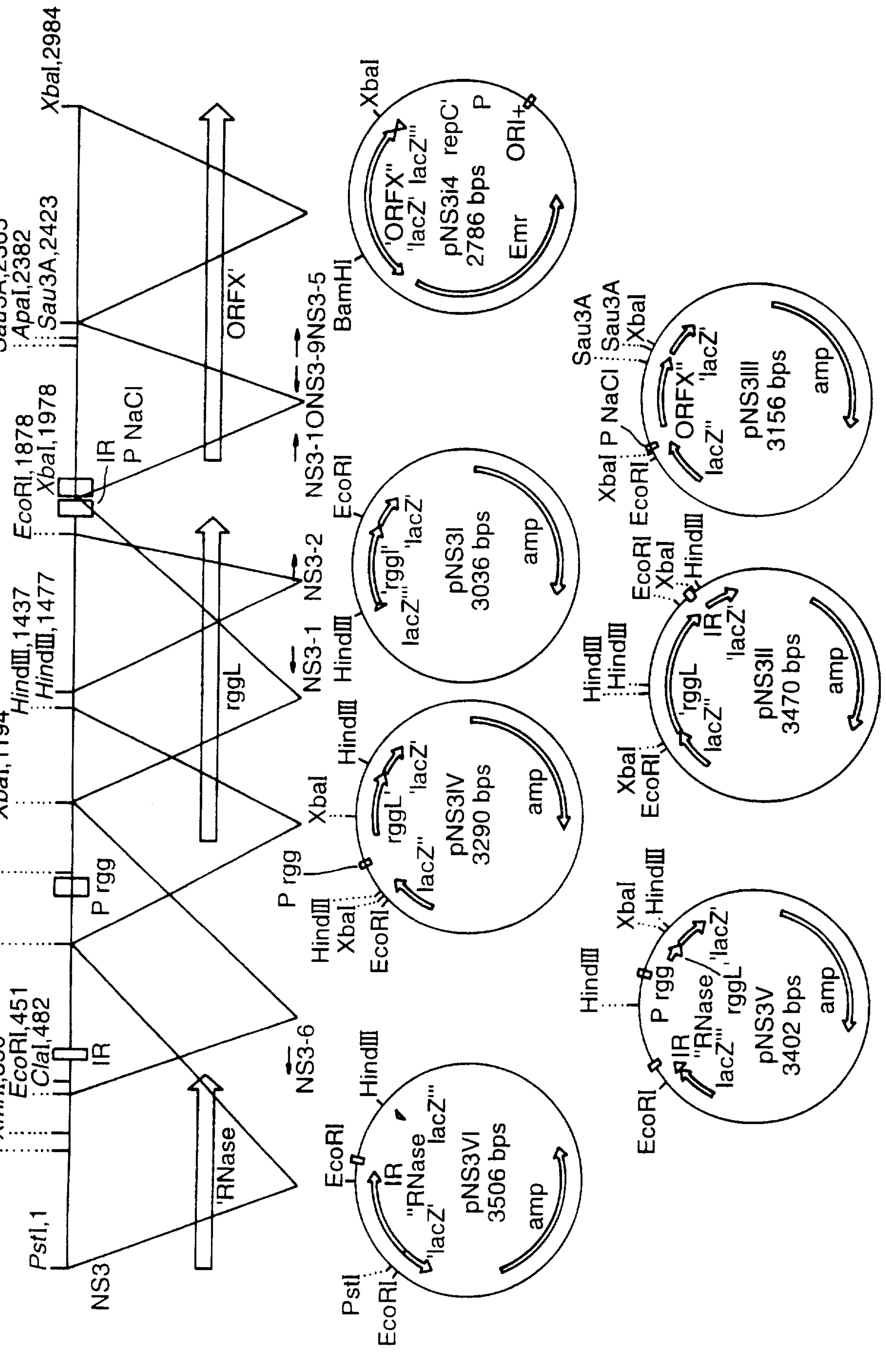
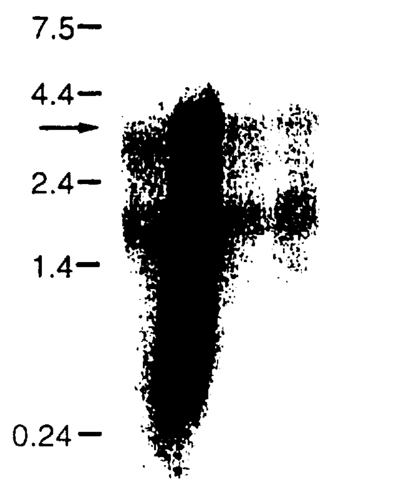
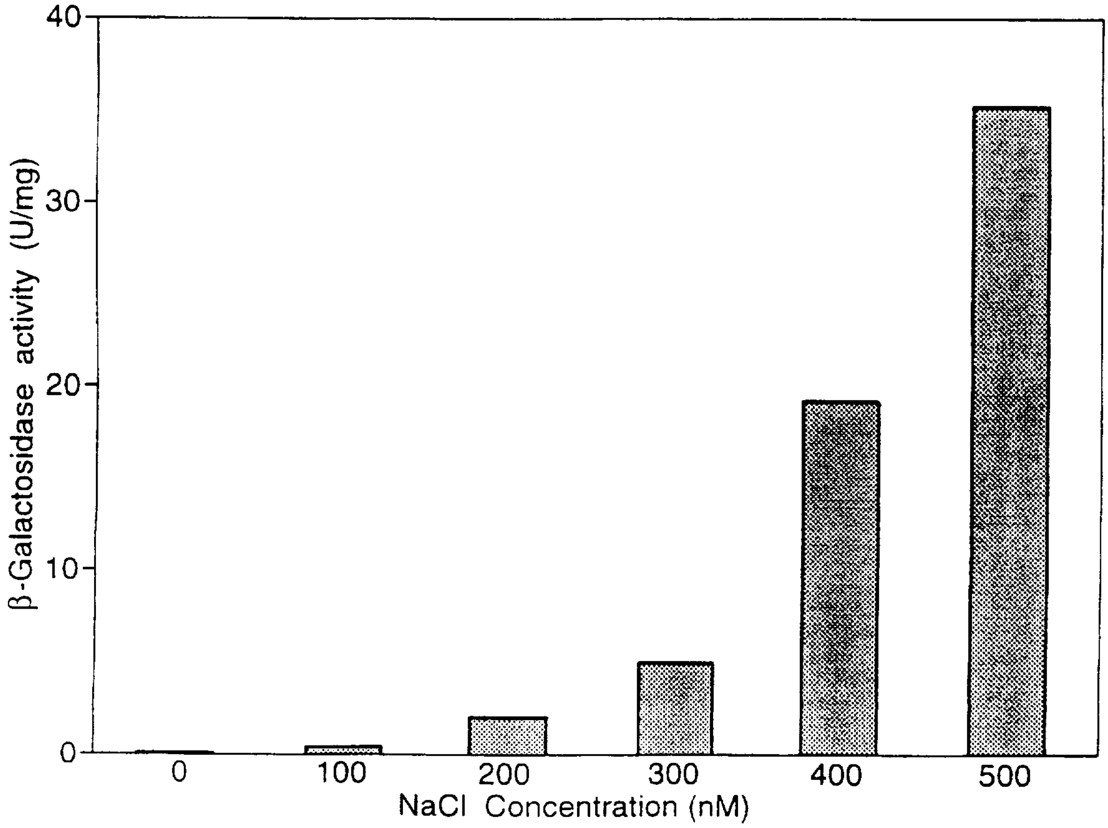

PstI,1
NotI,306
XmaIII,307
XmnI,350
EcoRI,451
ClaI,482
HindIII,833
AccI,1015
XbaI,1194
HindIII,1437
HindIII,1477
EcoRI,1878
XbaI,1978
Sau3A,2365
ApaI,2382
Sau3A,2423
XbaI,2984
NS3
IR
P rgg
IR
P NaCl
'RNase
rggL
'ORFX'
NS3-6
NS3-1
NS3-2
NS3-10
NS3-9
NS3-5
pNS3VI
3506 bps
pNS3IV
3290 bps
pNS3I
3036 bps
pNS3I4
2786 bps
pNS3V
3402 bps
pNS3II
3470 bps
pNS3III
3156 bps
amp
Emr
repC'
ORI+
BamHI
EcoRI
HindIII
XbaI
PstI
Sau3A
'lacZ'
lacZ''
"RNase
'rggL'
"ORFX"

Fig.10.

# Fig.11.


MG1363 MGNS3i3
– + – + NaCl
7.5
4.4
2.4
1.4
0.24

# Fig.12.


β-Galactosidase activity (U/mg)
40
30
20
10
0
0
100
200
300
400
500
NaCl Concentration (nM)

# Fig.13.

## Part A

```
     XbaI         -10                ↓ Transcription start
  1  TCTAGAGCAT ATACTAGAGG GTGAAGATAG GTTGTCTGAA GCATTATAAC TTGTCTTTTA

                                 RBS                 ScaI   EcoRV/ScaI
 61  AAAAATTCAA TCATAAATAT AAGGAGGTAT GatgAAGTAC TGGAT|ACTTA TCAATGATGA
                                     ORFX': MetLysTyr TrpIle Leu IleAsnAspGlu

121  ACCTTGGTTT GTCGGAAAAG ATGTAGCAAT TGCTATTGGT TACAAGAATT TCAGGGATGC
      ProTrpPhe ValGlyLys AspValAlaIle AlaIleGlu TyrLysAsn PheArgAspAla

181  TTTGAAATCT CATGTAAAAG ACAAATATAA GAGGGAGTCG GACAGCAGTG ATTGGTTCAA
      LeuLysSer HisValLys AspLysTyrLys ArgGluSer AspSerSer AspTrpPheAsn

                                                          RBS
241  CGACAATATA TTATTGGAAA CGAACTGCAT AAAAAATAAA AAATAGGAGA AAGAACatgA
      AspAsnIle LeuLeuGlu ThrAsnCysIle LysAsnLys Lys***
                                                             lytP: Met

301  AAACATTTTT TAAAGATATG GCAGAACGTG CCATTAAAAC ATTTGCACAA GCAATGATTG
    LysThrPhePhe LysAspMet AlaGluArg AlaIleLysThr PheAlaGln AlaMetIle
```

## Part B

```
     XbaI         -10                ↓ Transcription start
  1  TCTAGAGCAT ATACTAGAGG GTGAAGATAG GTTGTCTGAA GCATTATAAC TTGTCTTTTA

                                 RBS          ScaI
 61  AAAGATTCAA TCATAAATAT AAGGAGGTAT GatgAAGTAC TTATTATATT TTGTAATCTT
                                     ORFX': MetLysTyr LeuLeuTyr PheValIlePhe
                                            MetLys Ser TyrTyrIle Leu***

       RBS
121  TAGAAAGGTA ATTATTTatg CCAGTATCAC GTGTTAAAGT TAAAAATAGA CATTTAAAAA
      ArgLysVal IleIleTyr AlaSerIleThr Cys***
                  acmA: Met ProValSer ArgValLys ValLysAsnArg HisLeuLys
```

Fig.14.

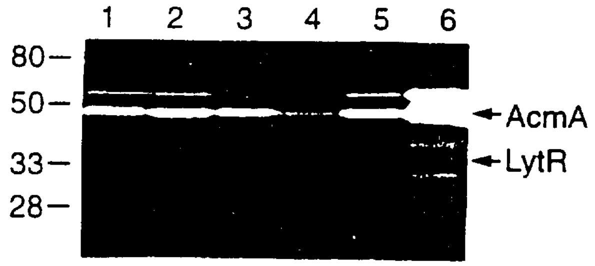

1 2 3 4 5 6
80
50
33
28
AcmA
LytR

Fig.15.

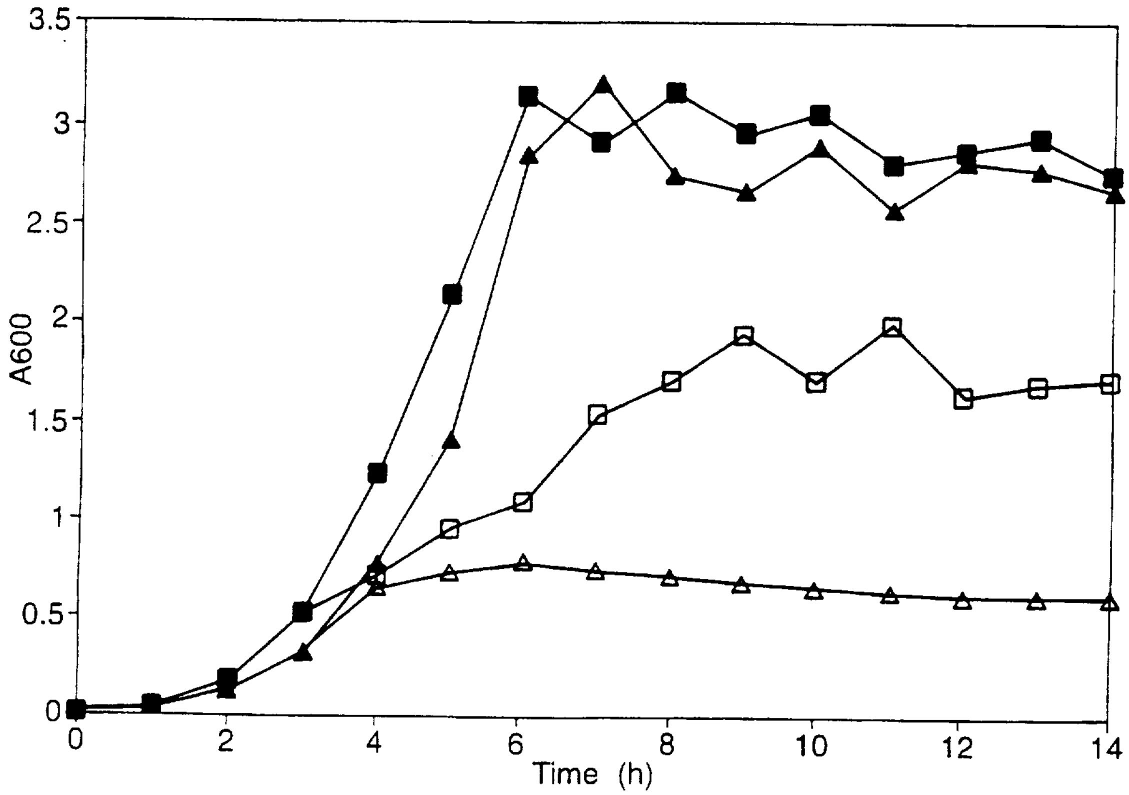

3.5
3
2.5
2
1.5
1
0.5
0
A600
0 2 4 6 8 10 12 14
Time (h)

Fig.16.

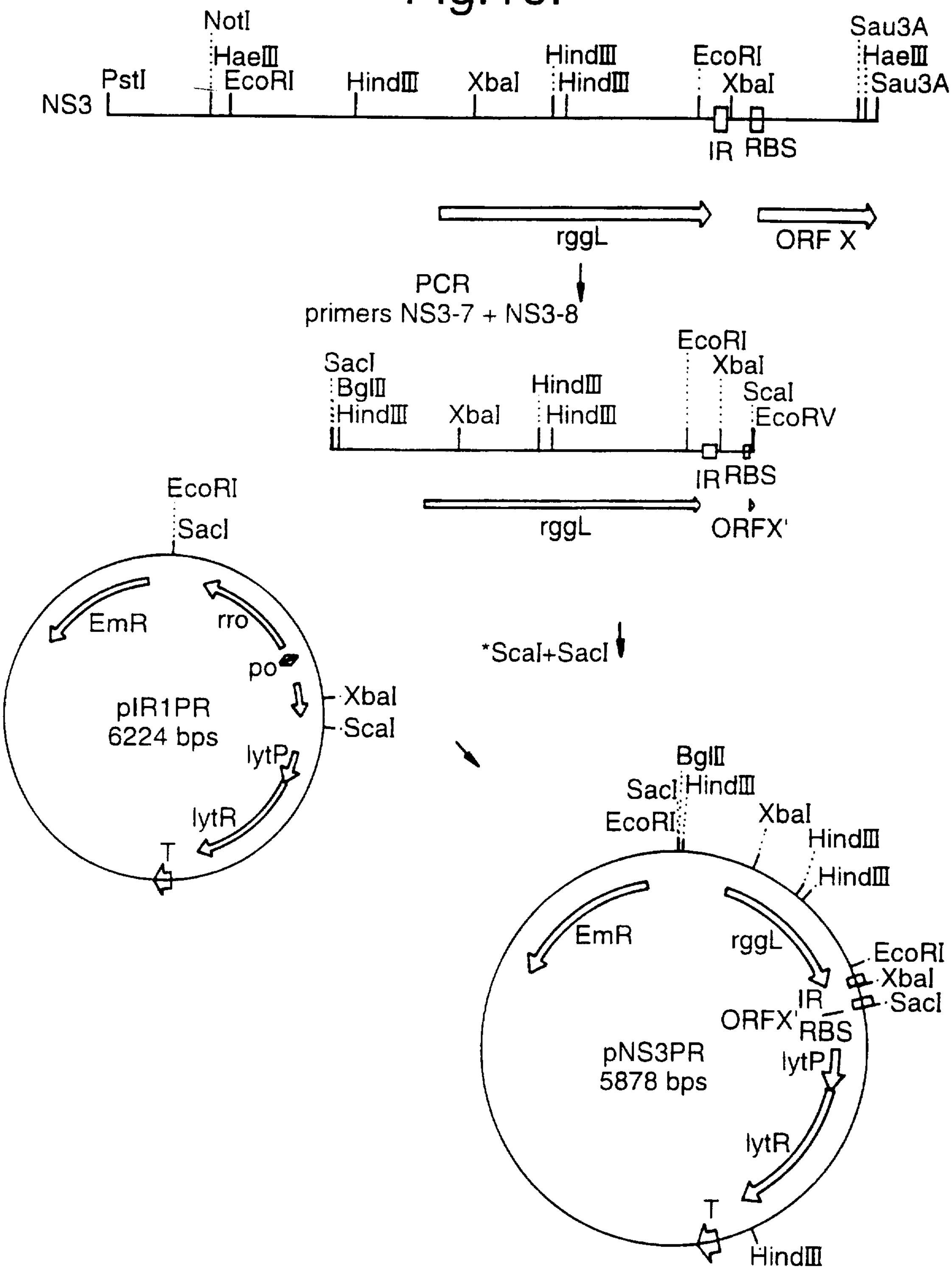

NotI
HaeIII
PstI
EcoRI
HindIII
XbaI
HindIII
HindIII
EcoRI
XbaI
Sau3A
HaeIII
Sau3A
NS3
IR
RBS
rggL
ORF X
PCR
primers NS3-7 + NS3-8
SacI
BglII
HindIII
XbaI
HindIII
HindIII
EcoRI
XbaI
ScaI
EcoRV
IR
RBS
rggL
ORFX'
EcoRI
SacI
EmR
rro
po
pIR1PR
6224 bps
XbaI
ScaI
lytP
lytR
T
*ScaI+SacI
BglII
SacI
HindIII
EcoRI
XbaI
HindIII
HindIII
EmR
rggL
EcoRI
XbaI
SacI
IR
ORFX'
RBS
pNS3PR
5878 bps
lytP
lytR
T
HindIII

Fig.17.

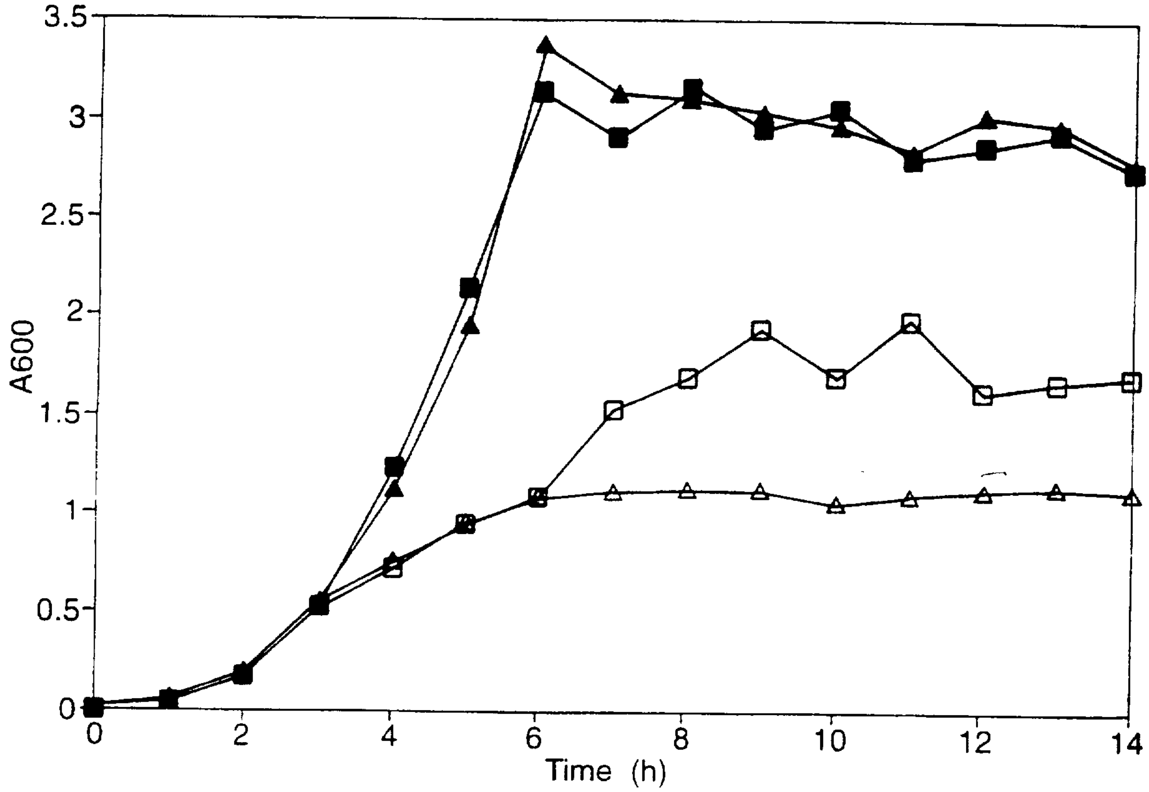

3.5
3
2.5
2
1.5
1
0.5
0
A600
0
2
4
6
8
10
12
14
Time (h)

Fig.19.

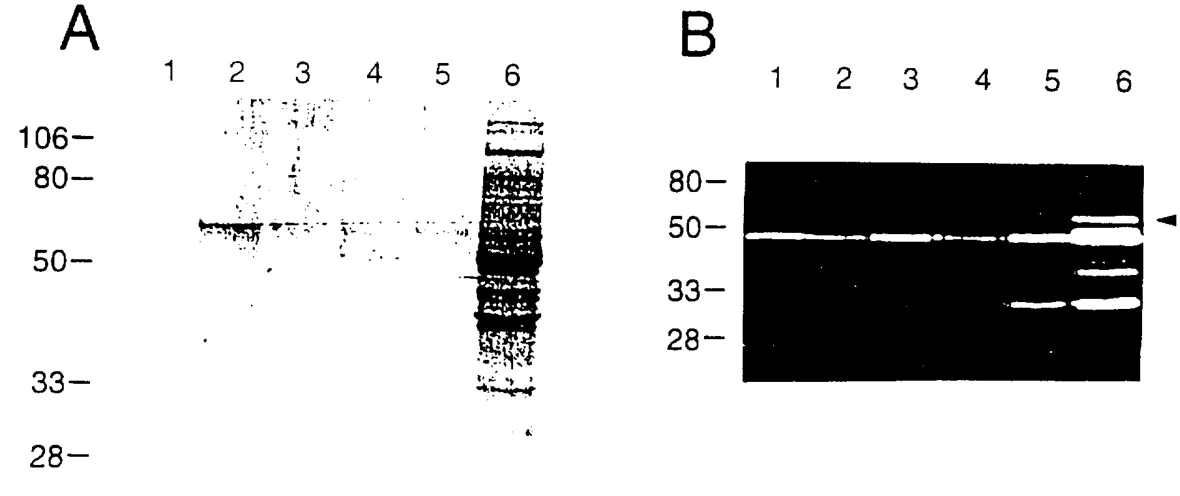

A
1 2 3 4 5 6
106—
80—
50—
33—
28—
B
1 2 3 4 5 6
80—
50—
33—
28—

Fig.18.

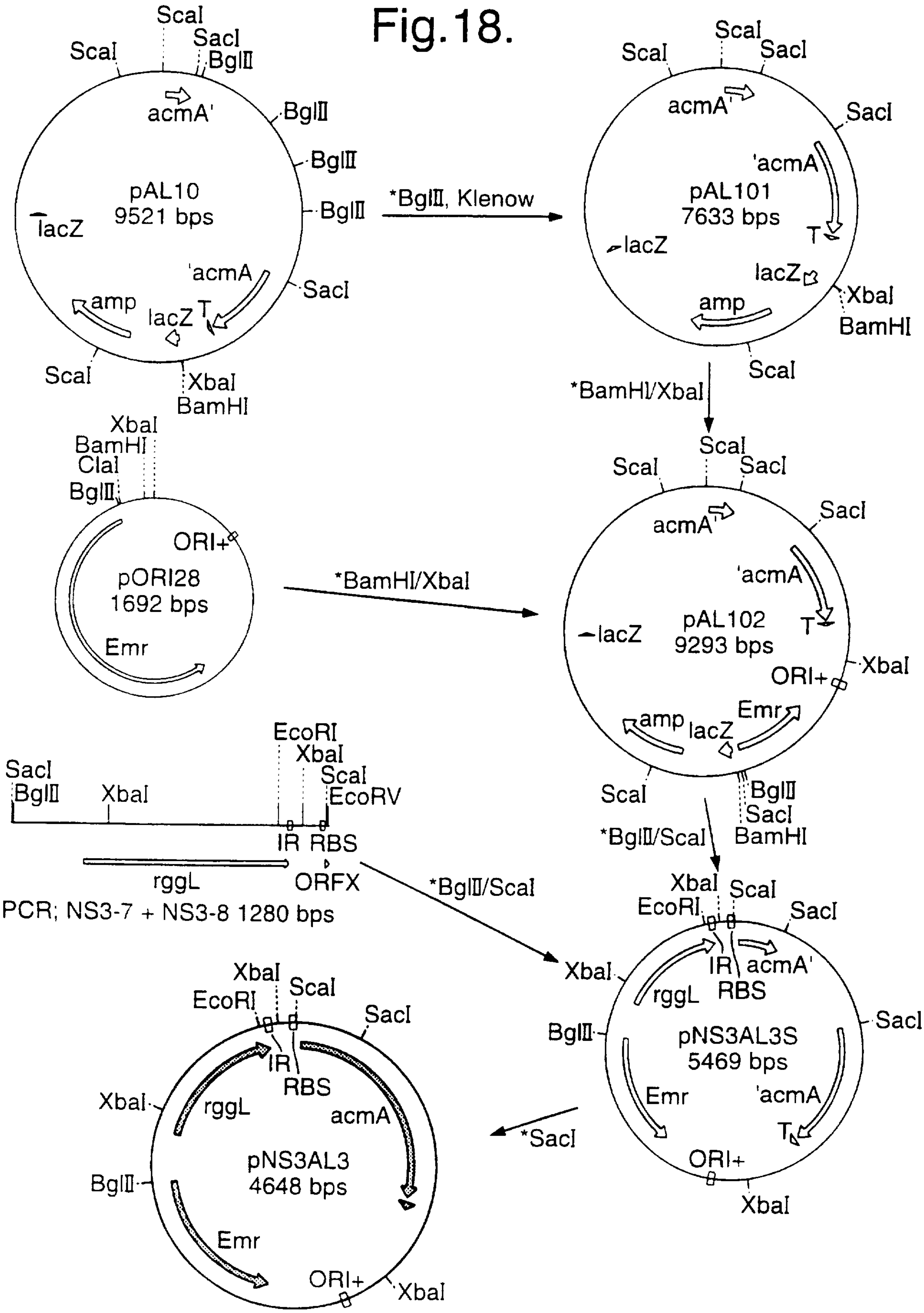

ScaI
ScaI
SacI
BglII
acmA'
BglII
BglII
BglII
pAL10
9521 bps
lacZ
'acmA
SacI
amp
lacZ
T
ScaI
XbaI
BamHI
*BglII, Klenow
ScaI
ScaI
SacI
acmA'
SacI
'acmA
pAL101
7633 bps
T
lacZ
lacZ
XbaI
BamHI
amp
ScaI
*BamHI/XbaI
XbaI
BamHI
ClaI
BglII
ORI+
pORI28
1692 bps
Emr
*BamHI/XbaI
ScaI
ScaI
SacI
acmA'
SacI
'acmA
lacZ
pAL102
9293 bps
T
XbaI
ORI+
Emr
amp
lacZ
ScaI
BglII
SacI
BamHI
*BglII/ScaI
EcoRI
XbaI
SacI
ScaI
BglII
XbaI
EcoRV
IR RBS
rggL
ORFX
PCR; NS3-7 + NS3-8 1280 bps
*BglII/ScaI
XbaI
ScaI
EcoRI
SacI
XbaI
IR
acmA'
rggL
RBS
BglII
SacI
pNS3AL3S
5469 bps
Emr
'acmA
T
ORI+
XbaI
*SacI
XbaI
EcoRI
ScaI
SacI
IR
RBS
XbaI
rggL
acmA
pNS3AL3
4648 bps
BglII
Emr
ORI+
XbaI

Fig.20.

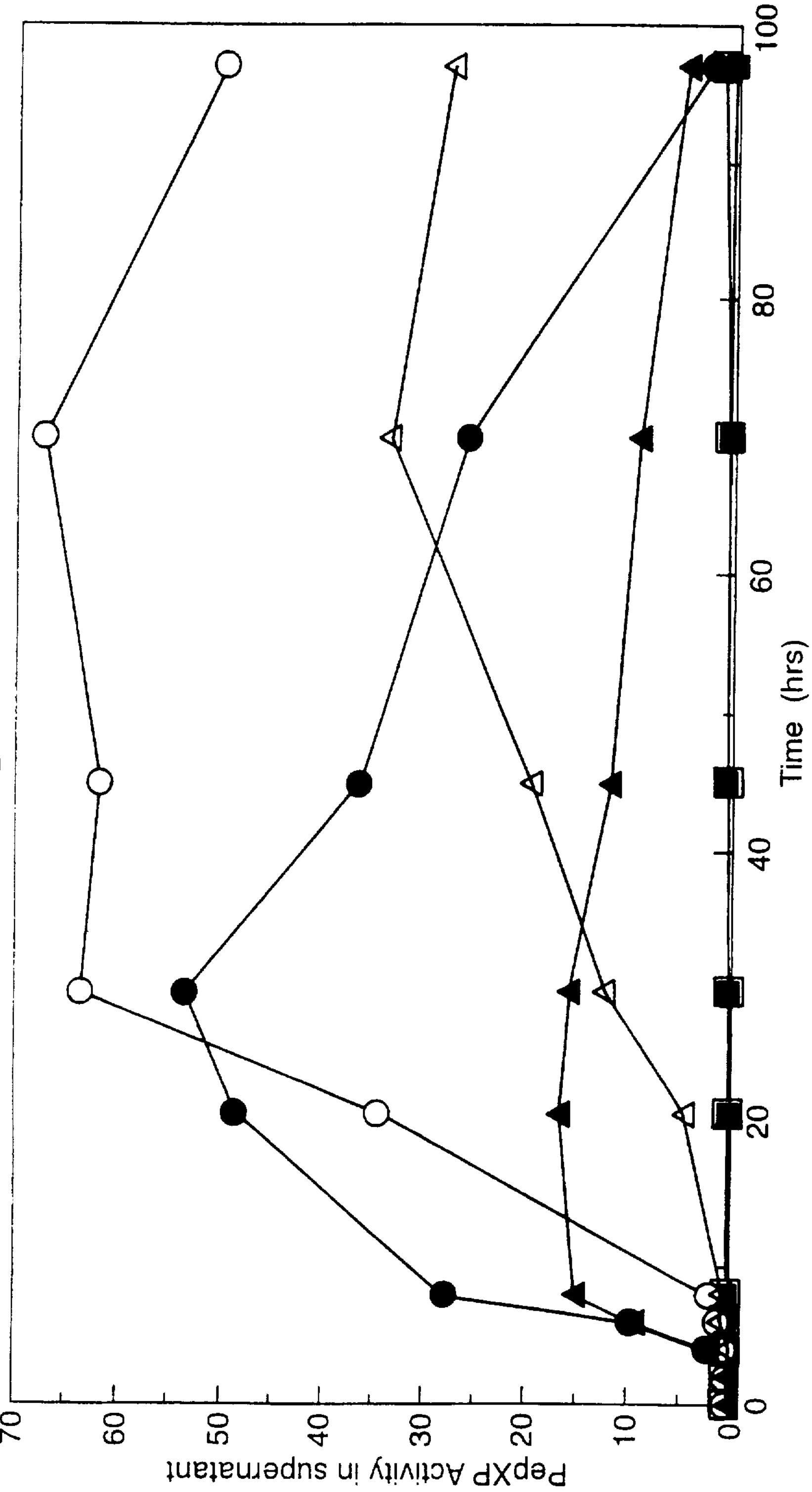
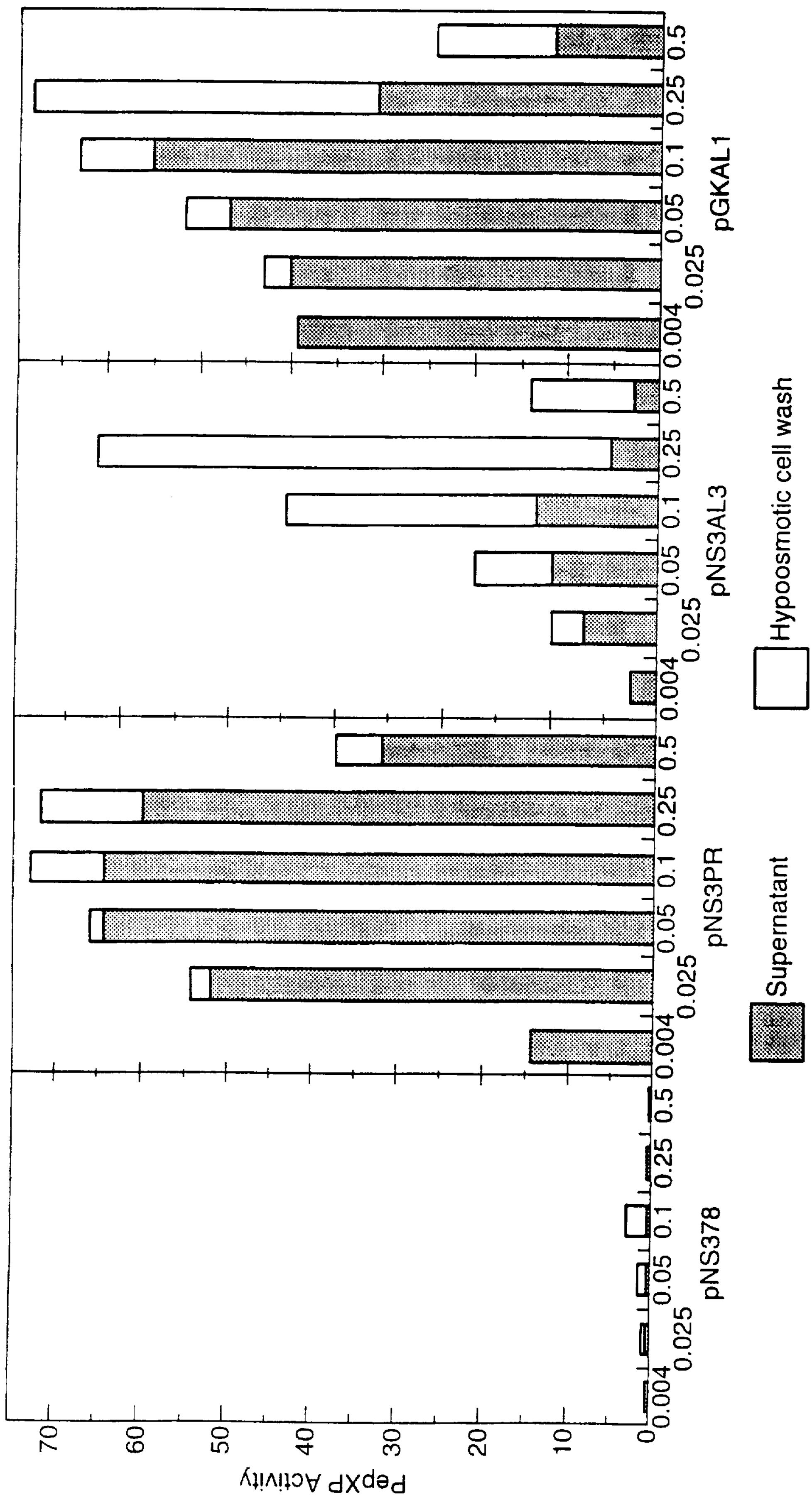

PepXP Activity in supernatant
0
10
20
30
40
50
60
70
0
20
40
60
80
100
Time (hrs)

Fig.21.


PepXP Activity
0
10
20
30
40
50
60
70
0.004
0.025
0.05
0.1
0.25
0.5
pNS378
pNS3PR
pNS3AL3
pGKAL1
Supernatant
Hypoosmotic cell wash

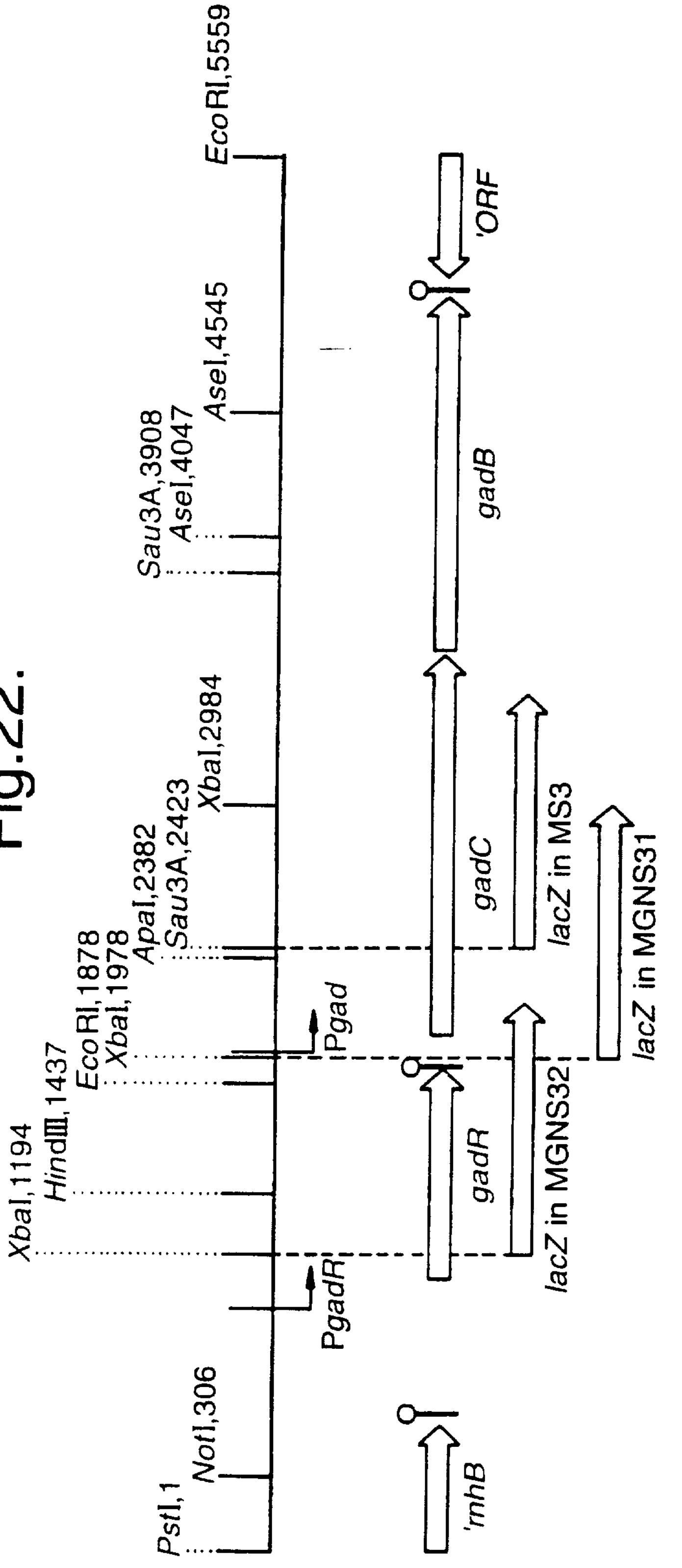

PstI,1
NotI,306
XbaI,1194
HindIII,1437
EcoRI,1878
XbaI,1978
ApaI,2382
Sau3A,2423
XbaI,2984
Sau3A,3908
AseI,4047
AseI,4545
EcoRI,5559
PgadR
Pgad
'rnhB
gadR
gadC
gadB
'ORF
lacZ in MGNS32
lacZ in MS3
lacZ in MGNS31

Fig.22.

# Fig.23.

```
L.lactis   M-------NQKKLSLFGFFALTASMVLTVYEYPTFATSKLHLVFFLLLGG   43
           *         * *.* ****.*****..**********   *********
Shigella   MATSVQTGKAKQLTLLGFFAITASMVMAVYEYPTFATSGFSLVFFLLLGG   50

L.lactis   LLWFLPVALCAAEMATVEGWKNGGIFSWVSQTLGERFGFAAIFFQWFQIT   93
           .***.** ********* **  **.*.***.*** *.***** * . **.
Shigella   ILWFIPVGLCAAEMATVDGWEEGGVFAWVSNTLGPRWGFAAISFGYLQIA  100

L.lactis   VGFVTMIYFILGALSYVLNFQALNTDPLIKFIGLLIIFWGLTFSQLGGTQ  143
           .**. *.**.******.*   *** **. * *  ***.* *  .* ***
Shigella   IGFIPMLYFVLGALSYILKWPALNEDPITKTIAALIILWALALTQFGGTK  150

L.lactis   RTAKLVKAGFVVGIVIPSVILFGLAAAYFIGGNPIEIPINSHAFVPDFSQ  193
            **.. * **  **..*. **  *** *   *  . * . * .* ****
Shigella   YTARIAKVGFFAGILLPAFILIALAAIYLHSGAPVAIEMDSKTFFPDFSK  200

L.lactis   VSTLVVFVSFILAYMGVEASASHINELENPKRNYPLAMILLVILAISLDA  243
           * ******.** .********.*.**. ** *.*****.**.. ** * .
Shigella   VGTLVVFVAFIGSYMGVEASATHVNEMSNPGRDYPLAMLLLMVAAICLSS  250

L.lactis   IGGFSVAAVIPQKELSLSAGVIQTFQTLILHFNHHLGWLVKVIALMIAFG  293
           .** *.* ***  *. *****.***  *. *    . *   **. ..  *
Shigella   VGGLSIAMVIPGNEINLSAGVMQTFTVLMSHVAPEIEWTVRVISALLLLG  300

L.lactis   VMGEVSSWVVGPSRGMFAAAQRGLLPKFLRKTNTHEVPVPLVMIQGIIVT  343
           *. *..**.*******.  **. ***    * *   *** **. * .* .
Shigella   VLAEIASWIVGPSRGMYVTAQKNLLPAAFAKMNKNGVPVTLVISQLVITS  350

L.lactis   LWGAVLTFGGGGNNLSFLVAISLTVVIYLVGYLLFFIVYFVLIYKKQNLK  393
           .   .**  *****.***.*..*******  * . ** * **. *   **
Shigella   IALIILTNTGGGNNMSFLIALALTVVIYLCAYFMLFIGYIVLVLKHPDLK  400

L.lactis   RTYNVPGKIIGKTIIAGIGFLLSIFALFISFVPPASIAKNETHTYQMILL  443
           **.*.**    * ..* .* * ** *  .**.**  *    *  *  .*
Shigella   RTFNIPGGKGVKLVVAIVGLLTSIMAFIVSFLPPDNIQGDSTDMYVELLV  450

L.lactis   ISFVVTAILPFIIYELHDKKGHD----TIEEPTHFKAGDVNPAIYPAARG  489
           .**.*   ****.* .**.**      *.*      *      . * **
Shigella   VSFLVVLALPFILYAVHDRKGKANTGVTLEPINSQNAPKGHFFLHPRARS  500

L.lactis   EHHIIKKEEHILKH   503
            * *.   .    **
Shigella   PHYIVMNDK---KH   511
```

# Fig.24.

```
L.lactis GadB     MLYGKENRD---EAE-FLEPIFGSESEQVDLPKYKLAQQSIEPRVAYQLV 46
                  *.  *       ***  * * . .      . ** .    . * .**.*.
Synechocystis     MVHKKIDLNQLSEAESLLTPTYAARGLANSVSKYEMPETEMLPAIAYNLI 50

L.lactis          QDEMLDEGNARLNLATFCQTYMEPEAVKLMSQTLEKNAIDKSEYPRTTEI 96
                   **.  .**.*******  *.*****  **. * .** *** *** *.**
Synechocystis     HDELGLDGNSRLNLATFVTTWMEPEARQLMADTFDKNMIDKDEYPQTAEI 100

L.lactis          ENRCVNMIADLWNASEKGKIYGTSTIGSSEACMLGGMAMKFSWRKRAEKL 146
                  * ****... ****       * ******** ********. ** *  .
Synechocystis     ELRCVNILSRLWNAPASAEATGCSTIGSSEAAMLGGMAMKWKWRQR--RQ 148

L.lactis          GLDINAKKPNLVISSGYQVCWEKFCVYWDIEMREVPMDREHMSINLEKVM 196
                         .****.    ******** **..* * ***. .   *  *  .
Synechocystis     AAGKPGDRPNLVMGINVQVCWEKFCRYWEVEPRFVPMEGDRYHISPEEAV 198

L.lactis          DYVDEYTIGVVGIMGITYTGRYDDIKALDNLIEEYNKQTDYKVYIHVDAA 246
                    .** ****.**.* *.* *. * **   .*  *  *  .* .*.***
Synechocystis     KLIDENTIGVIGILGSTFDGSYEPIEALNDALETLNQRTGWQVPLHIDAA 248

L.lactis          SGGLYAPFVEPELEWDFRLKNVISINTSGHKYGLVYPGVGWVLWRDKKYL 296
                  ***  ***..*.* *****  * ******************..****  *
Synechocystis     SGGFIAPFLDPDLRWDFRLPWVKSINTSGHKYGLVYPGVGWIIWRDKEEL 298

L.lactis          PEELIFKVSYLGGELPTMAINFSHSASQLIGQYYNFVRYGFDGYKAIHER 346
                  ******   ****.**  *.***    *.. *****.* * .**. *
Synechocystis     PEELIFHCNYLGGDLPNFALNFSRPGNQVVAQYYNFLRLGKEGYRKIQQT 348

L.lactis          THKVAMYLAEEIEKTGMFEIMNDGAQLPIVCYKLKEN--SNRGWNLYDLA 394
                      *.** *   * **.. **  .*.  ..**.    *  . ***.*
Synechocystis     CRDTALYLSGKIAQLGPFELLTDGGDIPVFAWRLKDEVLANTCYTLYDMA 398

L.lactis          DRLLMKGWQVPAYPLPKNLENEIIQRXXXRADFGMNMAFNYVQDMQEAID 444
                  *.*  .** **** .*** *  ..**   .  *   **   . **  **
Synechocystis     DKLRERGWLVPAYRMPKNREDLVVQRIVVKEGFSRDMADLLLADMERAIA 448

L.lactis          ALNKAHILFHQEPENKTYGFTH 466
                              * .    *.*
Synechocystis     YFASQP---DHKPKQEGSHFSH 467
```

Fig.25.

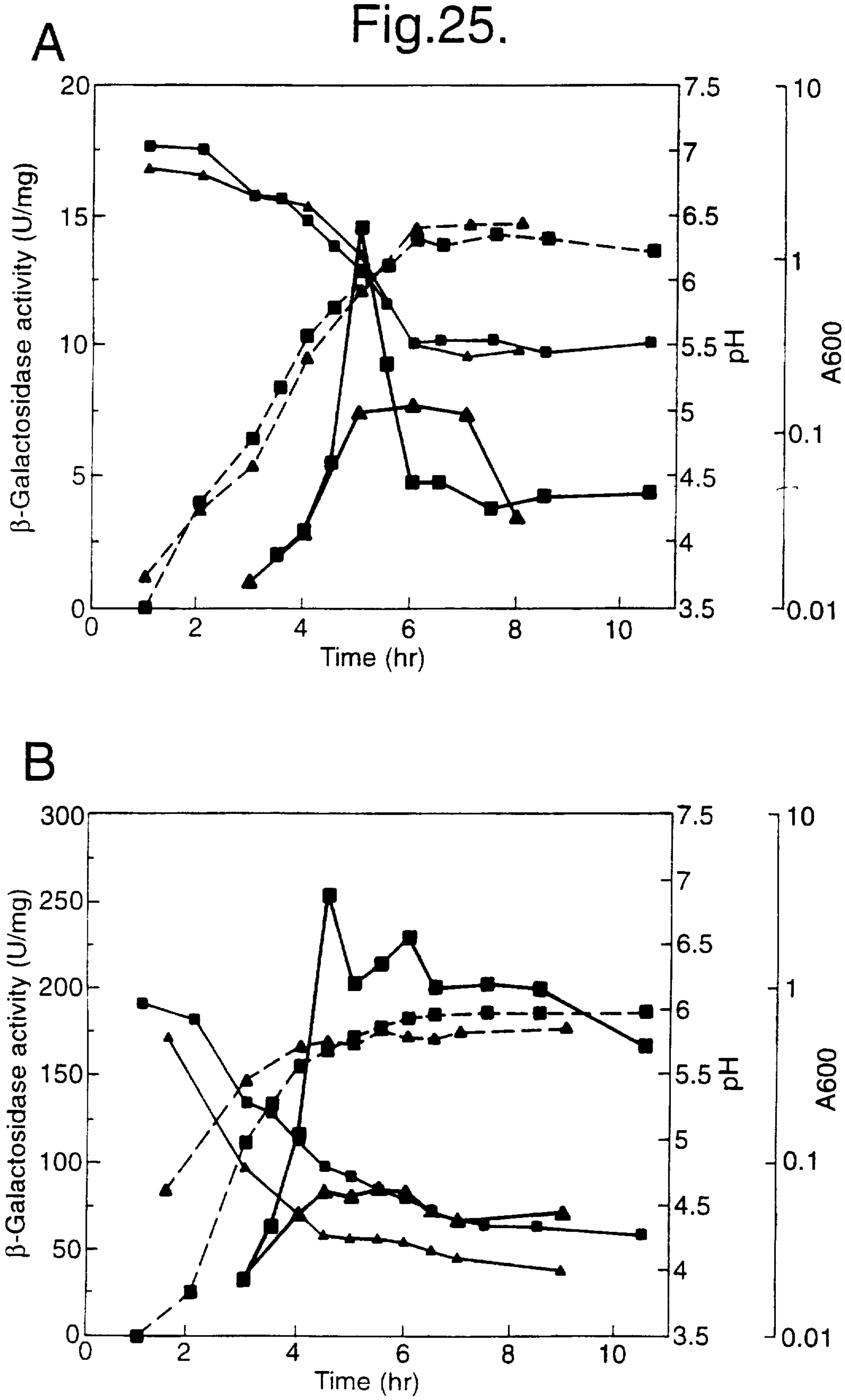
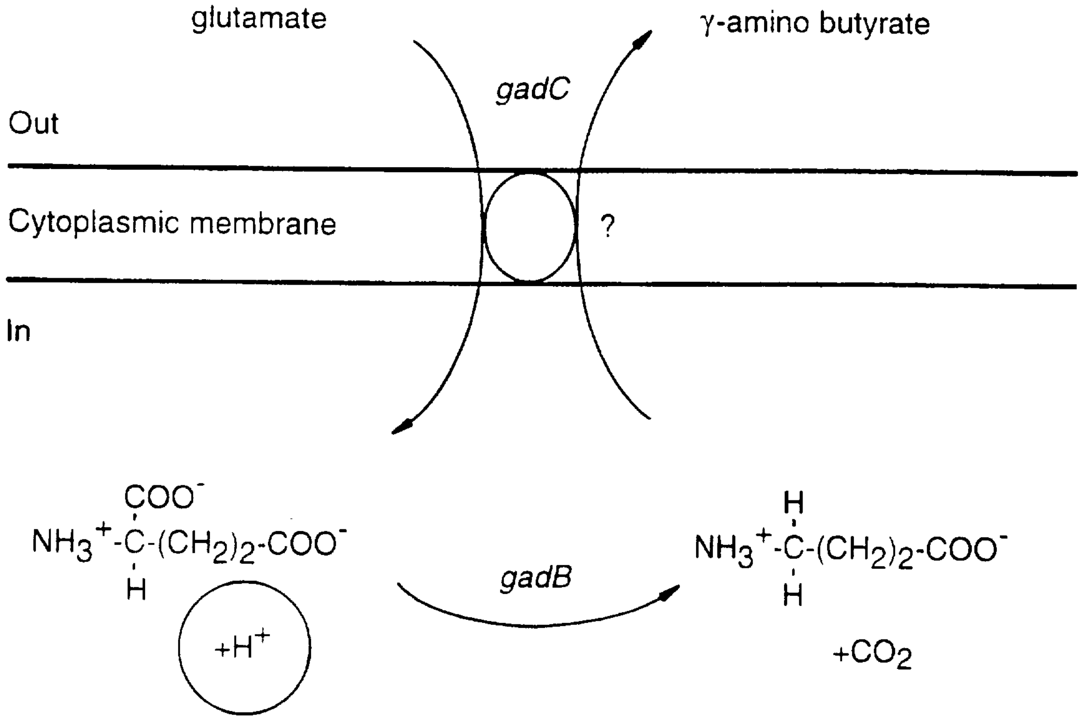

A
20
15
10
5
0
0
2
4
6
8
10
Time (hr)
β-Galactosidase activity (U/mg)
7.5
7
6.5
6
5.5
5
4.5
4
3.5
pH
10
1
0.1
0.01
A600
B
300
250
200
150
100
50
0
0
2
4
6
8
10
Time (hr)
β-Galactosidase activity (U/mg)
7.5
7
6.5
6
5.5
5
4.5
4
3.5
pH
10
1
0.1
0.01
A600

Fig.26.




glutamate decarboxylase

Fig.27.

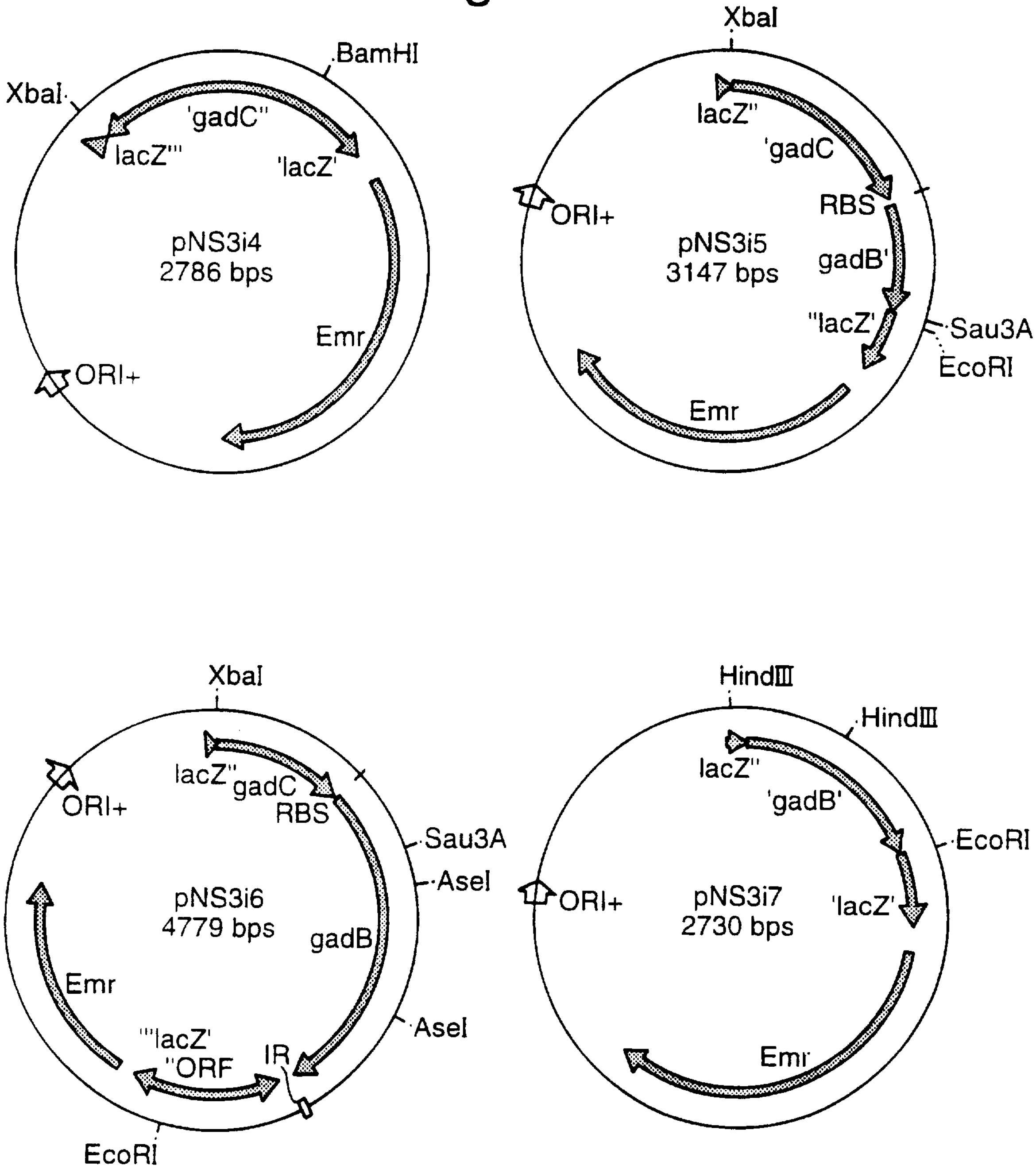
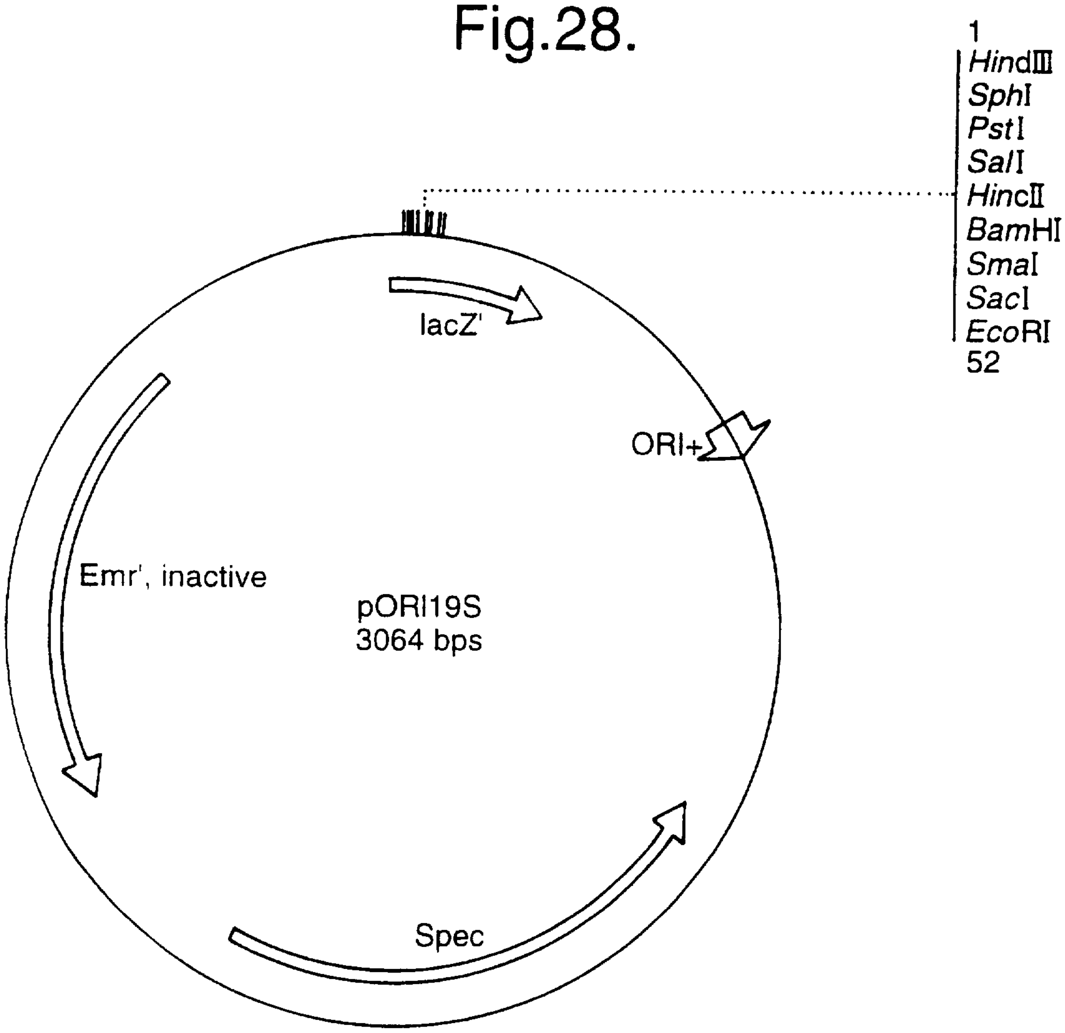

XbaI
BamHI
'gadC"
lacZ'''
'lacZ'
pNS3i4
2786 bps
Emr
ORI+
XbaI
lacZ"
'gadC
RBS
ORI+
pNS3i5
3147 bps
gadB'
"lacZ'
Sau3A
EcoRI
Emr
XbaI
ORI+
lacZ"
gadC
RBS
Sau3A
AseI
pNS3i6
4779 bps
gadB
Emr
'''lacZ'
"ORF
IR
AseI
EcoRI
HindIII
HindIII
lacZ"
'gadB'
EcoRI
ORI+
pNS3i7
2730 bps
'lacZ'
Emr

Fig.28.


1
*Hind*III
*Sph*I
*Pst*I
*Sal*I
*Hinc*II
*Bam*HI
*Sma*I
*Sac*I
*Eco*RI
52
lacZ'
ORI+
Emr', inactive
pORI19S
3064 bps
Spec

# Fig.29. (1/6)

```
  PstI    'rnhB
CT GCA GTA ATT TTG CCA AAG AAT TGT AAA ATT CGT GGT TTG AAT GAT       47
   Ala Val Ile Leu Pro Lys Asn Cys Lys Ile Arg Gly Leu Asn Asp       15

AGT AAA AAA GTG CCA AAA TCA AAG CAT CAT GCT ATT CTA TCT GAA ATT      95
Ser Lys Lys Val Pro Lys Ser Lys His His Ala Ile Leu Ser Glu Ile      31

CAA GAA AAA GCG CTA GCG ATT GGA GTT GGA ATT GTT GAT GCC GAA AAA     143
Gln Glu Lys Ala Leu Ala Ile Gly Val Gly Ile Val Asp Ala Glu Lys      47

ATT GAT GAA GTA AAT ATT TAT GAG GCG ACA AAA ATT GCA ATG ATT CAG     191
Ile Asp Glu Val Asn Ile Tyr Glu Ala Thr Lys Ile Ala Met Ile Gln      63

GCA GTA TCA AAA TTA TCT CTT AAA CCT GAA CAT CTC TTA ATA GAT GCG     239
Ala Val Ser Lys Leu Ser Leu Lys Pro Glu His Leu Leu Ile Asp Ala      79

ATG GTT TTA GAT TTG CCC ATT GCT CAG ACG AAA ATC ATT CAT GGA GAT     287
Met Val Leu Asp Leu Pro Ile Ala Gln Thr Lys Ile Ile His Gly Asp      95

                              NotI
GCT CGT TCA GCT TCA ATT GCG GCC GCA TCA ATT GTA GCT AAA GTG ACT     335
Ala Arg Ser Ala Ser Ile Ala Ala Ala Ser Ile Val Ala Lys Val Thr     111

CGT GAT GAA ATG ATG AAG GAT TTC GCT TTA GAA TTT CCA GAA TAT GAT     383
Arg Asp Glu Met Met Lys Asp Phe Ala Leu Glu Phe Pro Glu Tyr Asp     127

TTT GAA CAT AAT GCA GGC TAT GGA ACA GCA AAA CAT CTT GCA GCT CTG     431
Phe Glu His Asn Ala Gly Tyr Gly Thr Ala Lys His Leu Ala Ala Leu     143

ACA AAA TAT GGT ATC ACA AGA ATT CAT CGG AAA TCC TAT GAA CCA ATT     479
Thr Lys Tyr Gly Ile Thr Arg Ile His Arg Lys Ser Tyr Glu Pro Ile     159

AAA TCG ATG GTC AAT TTC AAA TAG TAGATTA TGTAAGTAAA AAAAGGAAAA       530
Lys Ser Met Val Asn Phe Lys ***                           ----      166

CGTGAACGAT ATTTGGTCAC GTTTTTTTGC TGACAAGTCT GTCAGTAATT ATTTTCAAAG   590
----- - >      < - ---- -----

GTTTCAAAAA TATAGTCTAG TAATTTGCTA GACTAATCTT CTGTTTTTTA ATAATAAACT   650

AATTTTTTGT TAATCTAAAT GACAAAATTA ATAAGCAGAG TTTTTTATAA AATTAGCTAC   710

TTATAAAAAA TTTGAAATTG GTATAGTTAA ATCTGTTATA ATTTCCAATA TTTTTTAATA   770

ATAATTATTT TAACAAAATA CTTATATCAA AACTCTTTCA AAGTATATAA TGAGCGTTGT   830

ATAAGCTTTT ATGTCTTTCT ATATCAACTT TTAATAGAAA TATAAAGTAA TATAAATGTT   890

                                                        -35
TTTATAATAA ATTATGTGAG ATATATTTTT TTGTCCGTAC TGGTATAGAT TTGACGATTA   950

                 -10          ↓
AGTCTTAAAT AAGTTATAAT CTCAATTGCG TAATTTCTTA AATACAGAAA TAACAACTAC  1010

ATTGGTAGAC TGATTAAAAA GTGTACTTGA TGAACTGTTA TAAACCTTAA AAAAATAAAA  1070

                rbs        start gadR
ATAATAGTTT GGGggaTgtT AAAGATG TAT AAA AAA TAT GGA GAT TGT TTT AAA  1124
                          Met Tyr Lys Lys Tyr Gly Asp Cys Phe Lys    10

AAG TTG CGA AAC CAA AAG AAT TTA GGG TTA TCA TAC TTT AGT AAA CTT    1172
Lys Leu Arg Asn Gln Lys Asn Leu Gly Leu Ser Tyr Phe Ser Lys Leu      26
```

# Fig.29. (2/6)

```
                                     XbaI
GGA ATA GAC CGT TCA AAT ATA TCT AGA TTT GAA CAT GGA AAA TGT ATG      1220
Gly Ile Asp Arg Ser Asn Ile Ser Arg Phe Glu His Gly Lys Cys Met        42

ATG AGT TTT GAG CGT ATA GAT TTG ATG TTA GAA GAA ATG CAA GTT CCG      1268
Met Ser Phe Glu Arg Ile Asp Leu Met Leu Glu Glu Met Gln Val Pro        58

TTA TCT GAG TAC GAA TTG ATT GTA AAT AAT TTT ATG CCG AAT TTC CAA      1316
Leu Ser Glu Tyr Glu Leu Ile Val Asn Asn Phe Met Pro Asn Phe Gln        74

GAA TTT TTT ATA TTA GAA TTG GAA AAA GCT GAA TTT AGC CAA AAT CGA      1364
Glu Phe Phe Ile Leu Glu Leu Glu Lys Ala Glu Phe Ser Gln Asn Arg        90

GAT AAA ATA AAA GAG TTG TAT TCT GAG GTC AAA GAA ACG GGG AAT CAT      1412
Asp Lys Ile Lys Glu Leu Tyr Ser Glu Val Lys Glu Thr Gly Asn His       106

                                        HindIII
TTA CTG ACG GTT ACC GTG AAA ACG AAG CTT GGG AAT ATA AGT CAG ACA      1460
Leu Leu Thr Val Thr Val Lys Thr Lys Leu Gly Asn Ile Ser Gln Thr       122

                           HindIII
GAA GTT AAA GAA ATT GAA GCT TAT CTT TGC AAT ATT GAA GAG TGG GGA      1508
Glu Val Lys Glu Ile Glu Ala Tyr Leu Cys Asn Ile Glu Glu Trp Gly       138

TAT TTT GAA CTT ACT TTA TTT TAT TTT GTA TCT GAT TAT CTC AAT GTC      1556
Tyr Phe Glu Leu Thr Leu Phe Tyr Phe Val Ser Asp Tyr Leu Asn Val       154

AAT CAA TTA GAA TTG CTG CTT TTT AAT TTT GAT AAA AGA TGT GAA AAT      1604
Asn Gln Leu Glu Leu Leu Leu Phe Asn Phe Asp Lys Arg Cys Glu Asn       170

TAC TGT AGA GTC TTA AAA TAT AGA AGG AGA CTA TTG CAA ATA GCC TAT      1652
Tyr Cys Arg Val Leu Lys Tyr Arg Arg Arg Leu Leu Gln Ile Ala Tyr       186

AAA AGT GTT GCG ATA TAC GCG GCT AAA GGA GAA AGA AAA AAA GCC GAA      1700
Lys Ser Val Ala Ile Tyr Ala Ala Lys Gly Glu Arg Lys Lys Ala Glu       202

AAT ATT TTA GAA ATG ACT AAA AAA TAT CGA ACT GTG GGA GTC GAT TTA      1748
Asn Ile Leu Glu Met Thr Lys Lys Tyr Arg Thr Val Gly Val Asp Leu       218

TAT TCA GAA GTA TTA AGA CAT CTT GCT AGA GCT ATC ATT ATT TTT AAT      1796
Tyr Ser Glu Val Leu Arg His Leu Ala Arg Ala Ile Ile Ile Phe Asn       234

TTT GAA AAT GCA GAG ATT GGG GAA GAA AAA ATA AAT TAT GCT CTT GAG      1844
Phe Glu Asn Ala Glu Ile Gly Glu Glu Lys Ile Asn Tyr Ala Leu Glu       250

                                                EcoRI
ATT TTG GAA GAA TTT GGA GGA AAG AAG ATA AAA GAA TTC TAT CAG AAT      1892
Ile Leu Glu Glu Phe Gly Gly Lys Lys Ile Lys Glu Phe Tyr Gln Asn       266

AAA ATG GAA AAG TAT TTG AAA AGG TCA ATT TAG TCTCTTTTGA GCTGTTGCTT    1945
Lys Met Glu Lys Tyr Leu Lys Arg Ser Ile *** ---------- ----------     276

                                   XbaI       -10        ↓
TAAAGCAACA GCTCAAAAGA GATTTTCTTT ATTCTAGAGC ATATACTAGA GGGTGAAGAT    2005
><-------- ---------- --

                                                           rbs
GTCTGAAGCA TTATAACTTG TCTTTTAAAA AGGTTAATTC AATCATAAAT Ataaggaggt    2065

   Start gadC
ATG ATG AAT CAA AAA AAA TTA TCA TTA TTC GGT TTT TTC GCA TTA ACC      2113
    Met Asn Gln Lys Lys Leu Ser Leu Phe Gly Phe Phe Ala Leu Thr        15
```

# Fig.29. (3/6)

```
GCT TCA ATG GTT TTG ACT GTC TAT GAG TAT CCG ACT TTT GCC ACG TCA    2161
Ala Ser Met Val Leu Thr Val Tyr Glu Tyr Pro Thr Phe Ala Thr Ser      31

AAA TTA CAT TTG GTG TTC TTT TTA CTT CTC GGA GGA CTA CTA TGG TTT    2209
Lys Leu His Leu Val Phe Phe Leu Leu Leu Gly Gly Leu Leu Trp Phe      47

TTG CCT GTA GCG CTC TGC GCA GCA GAA ATG GCG ACG GTT GAA GGC TGG    2257
Leu Pro Val Ala Leu Cys Ala Ala Glu Met Ala Thr Val Glu Gly Trp      63

AAA AAT GGT GGA ATC TTT AGT TGG GTC AGT CAA ACT TTA GGT GAG CGC    2305
Lys Asn Gly Gly Ile Phe Ser Trp Val Ser Gln Thr Leu Gly Glu Arg      79

TTT GGT TTT GCA GCC ATA TTT TTT CAG TGG TTC CAA ATT ACA GTA GGT    2353
Phe Gly Phe Ala Ala Ile Phe Phe Gln Trp Phe Gln Ile Thr Val Gly      95

                                        ApaI
TTT GTC ACT ATG ATC TAT TTC ATT TTA GGG GCC CTC TCT TAT GTG TTA    2401
Phe Val Thr Met Ile Tyr Phe Ile Leu Gly Ala Leu Ser Tyr Val Leu     111

                            Sau3A
AAT TTT CAG GCG CTC AAT ACA GAT CCA TTG ATA AAA TTT ATT GGT TTA    2449
Asn Phe Gln Ala Leu Asn Thr Asp Pro Leu Ile Lys Phe Ile Gly Leu     127

CTA ATC ATT TTT TGG GGA TTG ACT TTT TCT CAA TTA GGT GGG ACG CAA    2497
Leu Ile Ile Phe Trp Gly Leu Thr Phe Ser Gln Leu Gly Gly Thr Gln     143

CGG ACT GCC AAA TTA GTA AAA GCT GGC TTT GTA GTT GGA ATA GTG ATT    2545
Arg Thr Ala Lys Leu Val Lys Ala Gly Phe Val Val Gly Ile Val Ile     159

CCA TCG GTT ATC TTG TTT GGA TTA GCA GCG GCA TAC TTT ATC GGA GGC    2593
Pro Ser Val Ile Leu Phe Gly Leu Ala Ala Ala Tyr Phe Ile Gly Gly     175

AAT CCT ATA GAA ATA CCA ATT AAC AGC CAT GCT TTT GTA CCA GAT TTT    2641
Asn Pro Ile Glu Ile Pro Ile Asn Ser His Ala Phe Val Pro Asp Phe     191

TCA CAG GTA TCA ACT TTA GTA GTT TTT GTT TCT TTT ATT CTG GCT TAT    2689
Ser Gln Val Ser Thr Leu Val Val Phe Val Ser Phe Ile Leu Ala Tyr     207

ATG GGG GTA GAA GCC TCA GCT TCA CAT ATT AAT GAA CTT GAA AAT CCA    2737
Met Gly Val Glu Ala Ser Ala Ser His Ile Asn Glu Leu Glu Asn Pro     223

AAA CGA AAT TAT CCC TTA GCA ATG ATT TTA TTA GTA ATT TTG GCT ATT    2785
Lys Arg Asn Tyr Pro Leu Ala Met Ile Leu Leu Val Ile Leu Ala Ile     239

TCT TTA GAT GCC ATA GGT GGA TTT TCT GTA GCA GCA GTT ATT CCT CAA    2833
Ser Leu Asp Ala Ile Gly Gly Phe Ser Val Ala Ala Val Ile Pro Gln     255

AAA GAG TTA TCA TTA AGT GCA GGG GTA ATC CAA ACT TTT CAA ACG TTA    2881
Lys Glu Leu Ser Leu Ser Ala Gly Val Ile Gln Thr Phe Gln Thr Leu     271

ATC TTA CAT TTT AAT CAT CAT TTG GGA TGG TTA GTT AAA GTG ATT GCA    2929
Ile Leu His Phe Asn His His Leu Gly Trp Leu Val Lys Val Ile Ala     287

CTA ATG ATT GCC TTT GGG GTT ATG GGA GAA GTG AGT TCA TGG GTT GTT    2977
Leu Met Ile Ala Phe Gly Val Met Gly Glu Val Ser Ser Trp Val Val     303

        XbaI
GGT CCT TCT AGA GGG ATG TTT GCA GCA GCA CAA AGA GGT TTA TTA CCA    3025
Gly Pro Ser Arg Gly Met Phe Ala Ala Ala Gln Arg Gly Leu Leu Pro     319

AAA TTT TTA CGT AAA ACG AAT ACA CAT GAA GTC CCT GTT CCT TTA GTT    3073
Lys Phe Leu Arg Lys Thr Asn Thr His Glu Val Pro Val Pro Leu Val     335
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# Fig.29. (4/6)

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ATG ATT CAA GGA ATC ATT GTT ACA CTT TGG GGC GCT GTA TTA ACT TTT   3121
Met Ile Gln Gly Ile Ile Val Thr Leu Trp Gly Ala Val Leu Thr Phe    351

GGA GGA GGA GGA AAT AAT TTA TCT TTC TTA GTT GCC ATT TCA CTG ACT   3169
Gly Gly Gly Gly Asn Asn Leu Ser Phe Leu Val Ala Ile Ser Leu Thr    367

GTA GTG ATT TAT TTG GTG GGT TAC CTC TTG TTC TTT ATT GTT TAC TTT   3217
Val Val Ile Tyr Leu Val Gly Tyr Leu Leu Phe Phe Ile Val Tyr Phe    383

GTT TTA ATC TAT AAA AAA CAA AAT TTA AAG CGT ACT TAT AAT GTT CCA   3265
Val Leu Ile Tyr Lys Lys Gln Asn Leu Lys Arg Thr Tyr Asn Val Pro    399

GGT AAA ATA ATA GGA AAA ACA ATC ATT GCA GGA ATT GGA TTC TTA TTA   3313
Gly Lys Ile Ile Gly Lys Thr Ile Ile Ala Gly Ile Gly Phe Leu Leu    415

TCA ATT TTT GCT CTA TTT ATT TCC TTT GTT CCT CCA GCA TCA ATT GCG   3361
Ser Ile Phe Ala Leu Phe Ile Ser Phe Val Pro Pro Ala Ser Ile Ala    431

AAA AAT GAA ACT CAC ACC TAT CAA ATG ATA CTT CTT ATA AGT TTT GTT   3409
Lys Asn Glu Thr His Thr Tyr Gln Met Ile Leu Leu Ile Ser Phe Val    447

GTG ACC GCT ATC TTG CCA TTT ATT ATT TAT GAA TTG CAT GAT AAA AAG   3457
Val Thr Ala Ile Leu Pro Phe Ile Ile Tyr Glu Leu His Asp Lys Lys    463

GGA CAT GAT ACT ATT GAA GAA CCA ACA CAC TTT AAA GCA GGA GAT GTG   3505
Gly His Asp Thr Ile Glu Glu Pro Thr His Phe Lys Ala Gly Asp Val    479

AAC CCT GCG ATT TAT CCA GCA GCT CGT GGA GAG CAT CAT ATT ATT AAA   3553
Asn Pro Ala Ile Tyr Pro Ala Ala Arg Gly Glu His His Ile Ile Lys    495

                                                 rbs          start gadB
AAA GAA GAA CAT ATC TTA AAA CAT TGA AAAATTggag gATGTACAT ATG   3602
Lys Glu Glu His Ile Leu Lys His ***                          Met      1

TTA TAC GGA AAA GAA AAT CGA GAT GAA GCG GAG TTC TTG GAA CCA ATT   3650
Leu Tyr Gly Lys Glu Asn Arg Asp Glu Ala Glu Phe Leu Glu Pro Ile     17

TTT GGT TCA GAA AGT GAA CAA GTG GAT TTA CCT AAA TAT AAA TTA GCT   3698
Phe Gly Ser Glu Ser Glu Gln Val Asp Leu Pro Lys Tyr Lys Leu Ala     33

CAA CAA TCA ATT GAG CCT CGA GTG GCC TAT CAG TTA GTT CAA GAT GAA   3746
Gln Gln Ser Ile Glu Pro Arg Val Ala Tyr Gln Leu Val Gln Asp Glu     49

ATG CTA GAT GAA GGG AAC GCT CGT TTA AAT TTG GCC ACA TTC TGT CAA   3794
Met Leu Asp Glu Gly Asn Ala Arg Leu Asn Leu Ala Thr Phe Cys Gln     65

ACT TAT ATG GAA CCT GAA GCA GTC AAG CTG ATG AGT CAG ACC TTG GAA   3842
Thr Tyr Met Glu Pro Glu Ala Val Lys Leu Met Ser Gln Thr Leu Glu     81

AAA AAT GCG ATT GAC AAA TCA GAA TAT CCA AGA ACA ACT GAA ATT GAA   3890
Lys Asn Ala Ile Asp Lys Ser Glu Tyr Pro Arg Thr Thr Glu Ile Glu     97

AAC CGT TGC GTC AAC ATG ATC GCT GAC CTT TGG AAT GCG AGT GAA AAA   3938
Asn Arg Cys Val Asn Met Ile Ala Asp Leu Trp Asn Ala Ser Glu Lys    113

GGA AAA ATT TAT GGG ACT TCG ACA ATT GGT TCT TCA GAA GCT TGT ATG   3986
Gly Lys Ile Tyr Gly Thr Ser Thr Ile Gly Ser Ser Glu Ala Cys Met    129

CTT GGG GGA ATG GCT ATG AAG TTT TCT TGG CGT AAG CGA GCA GAA AAA   4034
Leu Gly Gly Met Ala Met Lys Phe Ser Trp Arg Lys Arg Ala Glu Lys    145

TTA GGC CTA GAT ATT AAT GCG AAA AAG CCA AAC TTA GTC ATT TCC TCT   4082
Leu Gly Leu Asp Ile Asn Ala Lys Lys Pro Asn Leu Val Ile Ser Ser    161
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# Fig.29. (5/6)

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GGT TAT CAA GTT TGC TGG GAA AAA TTC TGT GTT TAT TGG GAT ATT GAA    4130
Gly Tyr Gln Val Cys Trp Glu Lys Phe Cys Val Tyr Trp Asp Ile Glu    177

ATG AGA GAA GTG CCA ATG GAT AGA GAA CAT ATG TCA ATC AAT TTG GAA    4178
Met Arg Glu Val Pro Met Asp Arg Glu His Met Ser Ile Asn Leu Glu    193

AAA GTG ATG GAT TAT GTT GAT GAA TAT ACG ATT GGA GTA GTT GGA ATT    4226
Lys Val Met Asp Tyr Val Asp Glu Tyr Thr Ile Gly Val Val Gly Ile    209

ATG GGG ATT ACT TAT ACT GGT CGT TAT GAT GAT ATC AAA GCT TTG GAT    4274
Met Gly Ile Thr Tyr Thr Gly Arg Tyr Asp Asp Ile Lys Ala Leu Asp    225

AAT TTG ATT GAA GAA TAT AAT AAA CAG ACA GAC TAC AAA GTT TAT ATT    4322
Asn Leu Ile Glu Glu Tyr Asn Lys Gln Thr Asp Tyr Lys Val Tyr Ile    241

CAC GTA GAT GCT GCT TCA GGA GGA CTT TAT GCT CCT TTT GTT GAG CCA    4370
His Val Asp Ala Ala Ser Gly Gly Leu Tyr Ala Pro Phe Val Glu Pro    257

GAA CTT GAG TGG GAT TTC CGT TTG AAA AAT GTC ATT TCA ATC AAT ACT    4418
Glu Leu Glu Trp Asp Phe Arg Leu Lys Asn Val Ile Ser Ile Asn Thr    273

TCA GGA CAT AAA TAT GGT TTA GTA TAT CCT GGT GTA GGT TGG GTC TTG    4466
Ser Gly His Lys Tyr Gly Leu Val Tyr Pro Gly Val Gly Trp Val Leu    289

TGG CGT GAC AAA AAA TAT TTA CCT GAA GAG TTA ATT TTT AAA GTA AGT    4514
Trp Arg Asp Lys Lys Tyr Leu Pro Glu Glu Leu Ile Phe Lys Val Ser    305

TAT CTT GGA GGA GAA TTA CCA ACA ATG GCG ATT AAT TTT TCT CAC AGT    4562
Tyr Leu Gly Gly Glu Leu Pro Thr Met Ala Ile Asn Phe Ser His Ser    321

GCT TCT CAA TTA ATC GGT CAA TAC TAT AAT TTT GTA CGT TAT GGA TTT    4610
Ala Ser Gln Leu Ile Gly Gln Tyr Tyr Asn Phe Val Arg Tyr Gly Phe    337

GAT GGA TAT AAA GCT ATT CAT GAG AGA ACG CAT AAA GTA GCC ATG TAT    4658
Asp Gly Tyr Lys Ala Ile His Glu Arg Thr His Lys Val Ala Met Tyr    353

TTA GCA GAA GAA ATT GAA AAA ACA GGA ATG TTT GAG ATT ATG AAC GAT    4706
Leu Ala Glu Glu Ile Glu Lys Thr Gly Met Phe Glu Ile Met Asn Asp    369

GGG GCA CAA TTA CCA ATT GTC TGC TAC AAA TTA AAA GAA AAT TCA AAC    4754
Gly Ala Gln Leu Pro Ile Val Cys Tyr Lys Leu Lys Glu Asn Ser Asn    385

CGT GGT TGG AAT CTT TAT GAT TTG GCA GAT CGT TTA TTA ATG AAG GGA    4802
Arg Gly Trp Asn Leu Tyr Asp Leu Ala Asp Arg Leu Leu Met Lys Gly    401

TGG CAA GTG CCT GCT TAT CCA CTT CCT AAA AAT TTG GAA AAT GAA ATC    4850
Trp Gln Val Pro Ala Tyr Pro Leu Pro Lys Asn Leu Glu Asn Glu Ile    417

ATT CAA CGT TTA GTA ATT CGA GCA GAT TTC GGG ATG AAT ATG GCA TTT    4898
Ile Gln Arg Leu Val Ile Arg Ala Asp Phe Gly Met Asn Met Ala Phe    433

AAC TAT GTT CAA GAT ATG CAA GAA GCA ATT GAT GCA CTA AAT AAG GCT    4946
Asn Tyr Val Gln Asp Met Gln Glu Ala Ile Asp Ala Leu Asn Lys Ala    449

CAT ATT CTA TTT CAT CAG GAA CCT GAA AAT AAA ACA TAT GGC TTT ACT    4994
His Ile Leu Phe His Gln Glu Pro Glu Asn Lys Thr Tyr Gly Phe Thr    465

CAC TAA AGATAAAAGC GATATATCTA AGATATATCG CTTTTATTTT GTTTTAGGCT    5050
His ***   --------------->    <---------------                     466

ATTTACTAAT TAGCTTGTCG CTTA TTA TTT TTC ATA GTA TTT ATC CAA AAT    5101
                          *** Lys Glu Tyr Tyr Lys Asp Leu Ile     -8

TTC CAT TTT TAA AGG AGT AAT TTT AGA TAG GGG GGC AGT TAG ACT TGT    5149
Glu Met Lys Leu Pro Thr Ile Lys Ser Leu Pro Ala Thr Leu Ser Thr    -24
```

# Fig.29. (6/6)

```
TCT TAG GAA GAG CTT ATC TTC AAT GTT GAT GAT ACC CAG ATA TTT AAC     5197
Arg Leu Phe Leu Lys Asp Glu Ile Asn Ile Ile Gly Leu Tyr Lys Val      -40

TTG AGG GTA GTT AGC ATT GAC TTC TAT AAT TTG GGC TTT TTT CTC ACT     5245
Gln Pro Tyr Asn Ala Asn Val Glu Ile Ile Gln Ala Lys Lys Glu Ser      -56

AAT ATT TTC GTC TGT CAC GGG CAC ATC TAG GTT GAC CGT TCT TTC TTT     5293
Ile Asn Glu Asp Thr Val Pro Val Asp Leu Asn Val Thr Arg Glu Lys      -72

ATA AGA GTA ATT TTT GAG AGC AGC AAT ATT TCG GTT TGG AAT AAA AGT     5341
Tyr Ser Tyr Asn Lys Leu Ala Ala Ile Asn Arg Asn Pro Ile Phe Thr      -88

AGT CGC ACC GTC GGC TCC GAT AAC AGT AAT GGA ACG AAT TCC TAC GGT     5389
Thr Ala Gly Asp Ala Gly Ile Val Thr Ile Ser Arg Ile Gly Val Thr     -104

TTT CAC CAC TCC CTC AAT ATC AAG ACC GGC AAA GGC AAC CGT ATC CGC     5437
Lys Val Val Gly Glu Ile Asp Leu Gly Ala Phe Ala Val Thr Asp Ala     -120

AAC ATT GAT TTG ATG TTC AAC GAT AAT AAA GAA ACC ATT AAT GAT ATC     5485
Val Asn Ile Gln His Glu Val Ile Ile Phe Phe Gly Asn Ile Ile Asp     -136

AGC GAC TAA ATC TCG TCC AGC GAA ACC AAG AGC GAC TCC AAG GAT ACC     5533
Ala Val Leu Asp Arg Gly Ala Phe Gly Leu Ala Val Gly Leu Ile Gly     -152

                             'ORF       EcoRI
CGC GCC AGC TAG GAC ATT TGC AAC AGG AATTC                           5565
Ala Gly Ala Leu Val Asn Ala Val Pro Leu                             -162
```

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/04755

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C12N15/74 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 95 31563 A (QUEST INT ;NAUTA ARJEN (NL); VENEMA GERARD (NL); KOK JAN (NL); LED) 23 November 1995<br>cited in the application<br>see the whole document | 1,8-10 |
| A | WO 95 31562 A (QUEST INT ;NAUTA ARJEN (NL); VENEMA GERARD (NL); KOK JAN (NL); LED) 23 November 1995<br>see the whole document | 1 |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 16 January 1998 | 30. 01. 98 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Hillenbrand, G |

# INTERNATIONAL SEARCH REPORT

**Information on patent family members**

Intern. ..al Application No
PCT/EP 97/04755

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9531563 A | 23-11-95 | AU 2374395 A | 05-12-95 |
| | | EP 0759084 A | 26-02-97 |
| WO 9531562 A | 23-11-95 | AU 2354095 A | 05-12-95 |
| | | EP 0759999 A | 05-03-97 |